Goosecoid Regulates the Neural Inducing Strength of the Mouse Node

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The homeobox gene goosecoid was the first specific genetic marker of Spemann's organizer in vertebrate embryos to be discovered. In the frog, misexpression of this gene by RNA injection produces duplication of the posterior axis. For these reasons, the recent finding that mice lacking goosecoid function have no early axial defects was rather surprising. Here we assay the neural inducing strength of wild-type and goosecoid-mutant mouse nodes by transplantation into primitive streak stage chick embryos. Wild-type mouse nodes strongly induce the neural-specific transcription factors Sox2 and Sox3 in the chick host. Homozygous goosecoid+/− nodes are severely impaired in their ability to induce both genes. Heterozygous goosecoid+/− nodes induce Sox3 as well as wild-type nodes, but resemble −/− nodes in their limited ability to induce Sox2. We propose that goosecoid does play a role in regulating the neural inducing strength of the node and that regulative mechanisms exist which mask the early phenotypic consequences of goosecoid mutations in the intact mouse embryo.

INTRODUCTION

The organizer is a special region of gastrulating vertebrate embryos, defined by its ability to induce neural tissue when transplanted to an ectopic site. In amphibians, the organizer property is located at the dorsal lip of the blastopore and in amniotes, the organizer is Hensen's node (usually just called "the node" in mouse). The homeobox gene goosecoid was the first organizer-specific gene to be discovered (Cho et al., 1991; Blum et al., 1992; Izpisúa-Belmonte et al., 1993): when ectopically expressed by injection into the ventral side of an early Xenopus embryo, a supernumerary organizer is generated (Cho et al., 1991; Blum et al., 1992) and cell migration during gastrulation is affected (Niehrs et al., 1993); subsequently an almost complete secondary axis develops, suggesting that this gene is involved in defining the organizer region. Because of this information, two recently reported findings are surprising: first, the phenotype of mice lacking a functional goosecoid gene does not include obvious defects in gastrulation, neural induction, or any of the known early functions of the organizer (Yamada et al., 1995; Rivera-Pérez et al., 1995). Second, chick embryos from which all cells destined to form the organizer have been surgically removed regenerate a new organizer and subsequently develop normally, although the regenerated organizer does not express goosecoid (Psychoyos and Stern, 1996).

The chick embryo lends itself very well to assays of neural induction because a peripheral region, the area opaca, which does not normally contribute cells to any embryonic structure, can nevertheless respond to an organizer graft by generating neural tissue that is normally patterned and which expresses panneural markers (Streit et al., 1998). Here, we have made mouse-chick chimeras to assay the neural-inducing strength of nodes obtained from wild-type and goosecoid-mutant mouse embryos. We used chick-specific riboprobes to detect expression of the HMG-containing transcription factors Sox3 and Sox2, which are early, specific, and reliable markers expressed throughout the neural plate and neural tube of the chick embryo (Streit et al., 1998). We find that in the absence of goosecoid the neural-inducing ability of the mouse node is severely impaired in this assay. Even het-
Goosecoid and Neural Induction

TABLE 1
Numbers of Donor Embryos Recovered and Used for Sox2/3 Induction Experiments

<table>
<thead>
<tr>
<th>gsc genotype</th>
<th>N.o. dissected (exp.)</th>
<th>Dead</th>
<th>Lost</th>
<th>Too old</th>
<th>Other</th>
<th>N.o. used</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>55 (41)</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>+/-</td>
<td>65 (82)</td>
<td>6</td>
<td>3</td>
<td>14</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>+/-</td>
<td>44 (41)</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td>164</td>
<td>8</td>
<td>5</td>
<td>30</td>
<td>3</td>
<td>116</td>
</tr>
</tbody>
</table>

Note. All mice are β-gal-/-; the genotype for goosecoid is shown. The first column shows the number of embryos harvested, classified according to their genotype. The expected (exp.) number of embryos based on the Mendelian ratio (1:2:1) is shown in parentheses. The next three columns indicate the numbers of embryos that were not used because the donor tissue had died, the graft was lost, the donor was older than 0B stage, or the graft was accidentally placed in the wrong position in the host ("other"). The final column shows the numbers of embryos used for scoring.

Materials and Methods

Generation of β-Galactosidase (β-Gal)-Expressing Goosecoid Mutant Mouse Embryos

Goosecoid heterozygotes (Yamada et al., 1995) in a B6S JL/F1 background were crossed with the Rosa-26 mouse line (B6, 129-TgR) which expresses β-gal in all cells (Jackson Laboratories) to obtain goosecoid+/β-gal-/- mice. Mice of this genotype were back-crossed with Rosa-26 to obtain goosecoid+/β-gal-/- male mice. These were back-crossed with goosecoid+/β-gal-/- females to generate mouse embryos of different goosecoid genotypes for subsequent experiments.

Preparation of Donor Mouse Node

Embryos on the seventh day of gestation were recovered from the matings described above, staged as described by Downs and Davis (1993), and collected in M2 medium (Sigma) containing 10% fetal calf serum. The distal tip of the embryos was excised by mechanical dissection with siliconized glass needles. In some experiments, a piece of similar size was dissected from either the anterior or the posterior part of the same embryo and used as a control graft. The extraembryonic tissues were used to genotype each individual embryo by PCR, as previously described (Belo et al., 1998). The distribution of embryos according to genotype and the proportion of these that were used for induction experiments are shown in Table 1.

Transplantation of Mouse Node into Chick Host

Chick host embryos were staged according to Hamburger and Hamilton (1951). The dissected mouse node region was grafted into the area opaca of stage 3+4 chick embryos, at the level of the host node. The chimeric embryos were set up in modified N ew culture as previously described (Stern, 1993), except that mouse explant medium M2 plus 10% fetal calf serum was used instead of chick albumen in the culture. The grafted embryos were then cultured for 20–24 h at 38°C before analysis.

Visualization of β-Gal Activity and Whole Mount in Situ Hybridization

Chimeric embryos were fixed at room temperature in 4% paraformaldehyde (PFA) for 1 h. After fixation, they were rinsed in PBS several times before incubation at 30–32°C for 1–3 h in β-gal staining solution (0.4 mg/ml 5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside (Molecular Probes), 4 mM K₃Fe(CN)₆, 4 mM K₄Fe(CN)₆, 2 mM MgCl₂, 0.02% Nonidet P-40 in PBS), which yields a magenta color. After staining, embryos were postfixed in 4% PFA overnight at 4°C. Embryos were then assessed for the expression of the early panneural markers Sox3 and Sox2 (kind gifts from Drs. R. Lovell-Badge and P. Scotting) by whole mount in situ hybridization as previously described (Psychoyos and Stern, 1996; Streit et al., 1998). Digoxigenin-labeled riboprobes were transcribed from the 3'-untranslated region and were chick specific. The alkaline phosphatase reaction was visualized with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate to yield a purple/ blue color. Following in situ hybridization, the embryos were postfixed and embedded in paraffin wax for histological sectioning.

Results

The Grafted Mouse Node Survives and Develops in the Chick Host

In initial experiments, we grafted mouse nodes into the area opaca of chick host embryos and examined the morphology of the grafted node and surrounding host regions as well as the expression of a marker for the notochord of the mouse [mouse Sonic hedgehog (Shh)] after 24 h of culture. When the chick embryo culture was set up conventionally, using egg albumen as substrate, the mouse node did not
change morphology during culture and did not express Shh after culture (0/5). We reasoned that this could be due to the high pH of albumen (about 9.2), which is likely to be deleterious to the mouse tissue. For this reason we replaced the egg albumen culture medium with mouse M2 medium containing 10% fetal calf serum. These culture conditions allowed the grafted mouse node to survive, to express β-gal strongly, and to differentiate, elongating during the culture period and frequently forming a notochord that expressed Sonic hedgehog (9/10) (Fig. 1A).

The Mouse Node Induces the Early Neural Markers Sox3 and Sox2 in the Chick Host

Next, we assayed the ability of grafted E7.0 (Rosa-26) mouse nodes to induce neural markers in the area opaca of the chick host. Following culture, we stained the chimeras with β-gal staining solution (to reveal the grafted mouse cells) and then for either Sox2 or Sox3 (chick specific) by in situ hybridization, followed by histological sectioning of the embryos. A high proportion of chimeras (28/44; 64%) showed expression of the chick neural markers (Figs. 1B, 1C, 1E, and 1F), and the chick epiblast immediately above the grafted mouse node had elevated and thickened, similar to what is seen after a graft of a chick node (Storey et al., 1992; Streit et al., 1998) (Fig. 1E; F; inp). Sox3 was induced more frequently (86%) than Sox2 (44%). As controls, we grafted other portions of the egg cylinder: neither the anterior (0/12) nor the posterior region (0/6) induced Sox3 expression or columnar morphology in the chick host (Fig. 1D).

We also classified the results of this graft according to the stage of the donor mouse embryo. No statistically significant difference was found in the inducing ability of nodes obtained from mice at early streak (ES), mid-streak (MS), late streak (LS), or no allantoic bud (OB) stages, but nodes obtained from early bud (EB) stage embryos had reduced ability to induce Sox3 (1/9; 11%).

Goosecoid Mutant Nodes Have Impaired Ability to Induce Chick Neural Markers

The same experiment was done with nodes obtained from embryos derived from Rosa-26/goosecoid− crosses, and the genotype of each donor mouse node was determined retrospectively by PCR (Belo et al., 1998). The results are summarized in Table 2. Mouse nodes lacking both copies of goosecoid are greatly impaired in their ability to induce either marker. Nodes lacking one copy of goosecoid are able to induce Sox3 as well as wild-type nodes, but induce Sox2 much less efficiently (Table 2).

DISCUSSION

Our results show that the mouse node can induce the expression of the neural markers Sox3 and Sox2 in a host chick embryo. This was expected because other combinations of organizer and ectoderm across different vertebrate classes (including rabbit nodes placed into avian embryos and vice versa; Waddington, 1930, 1932, 1933, 1934; Waddington and Schmidt, 1933) can also induce neural tissue from the host (Waddington, 1930, 1932, 1933, 1934; Waddington and Schmidt, 1933; Blum et al., 1992; Kintner and Dodd, 1991; Hatta and Takahashi, 1996). Nodes obtained from older (late streak to no allantoic bud stages) mouse donor embryos generate ectopic expression of Sox2 at a slightly lower frequency than those from younger donors, but the difference is not apparent for Sox3, which appears to be an easier gene to induce overall (Table 2).

In addition, we show that lack of even a single copy of goosecoid reduces the ability of the mouse node to induce Sox2 and that lack of both copies impairs its ability to induce both genes. It is interesting to compare this finding with a previous report of crosses between goosecoid mutant mice and those defective in another gene expressed in the organizer, HNF3β. Double heterozygous (HNF3β/−/goosecoid/−) embryos have a severe early phenotype even though neither heterozygote alone shows any early defects (Filosa et al., 1997).

When assaying the neural inducing ability of the mouse node, consideration should be given to a contribution from anterior visceral endoderm (AVE), a tissue that been shown to be important for the induction and/or patterning of anterior regions of the nervous system (Thomas and Beddington, 1996; Varlet et al., 1997; Belo et al., 1997). Goosecoid is expressed in the AVE in addition to the node and may play a role in both tissues. Between the early and late streak stages, the AVE appears to move anteriorly such that at the early streak stage, the goosecoid expression domain overlaps the distal tip of the egg cylinder, while at late streak/no bud stage it has separated from the node. We might therefore expect to find differences in inducing ability of nodes (distal tips) dissected from early (ES–MS) stages compared to late (LS–OB) embryos. However, this is not the case (Table 2); in our assay, the distal tips of embryos from ES to OB stages are able to induce both Sox3 and Sox2.

More surprisingly, our assay reveals a requirement for goosecoid in the mouse node that is not apparent in the intact mouse embryo, where lack of even both copies of the gene does not affect early development (Yamada et al., 1995; Rivera-Pérez et al., 1995). It has been suggested that the lack of an early phenotype in the whole mutant animal could be due to functional redundancy with other goosecoid-related genes (Belo et al., 1998; Lemaire et al., 1997; Funke et al., 1997). In chick embryos, the goosecoid-related gene GSX (Lemaire et al., 1997) is expressed in the primitive streak but not in the organizer, suggesting that goosecoid expression represses GSX expression in the same cells (Lemaire et al., 1997). If the mouse has a gene homologous to GSX, one possibility is that the absence of goosecoid expression in the node allows GSX expressing cells to
take on node functions and compensate for the lack of goosecoid. In grafts of the node to a region remote from the endogenous domain of GSX expression, as done here, no such regulation would occur because of the absence of a population of GSX-expressing cells close to the graft. A second, and perhaps more likely, explanation is that...
TABLE 2

Induction of Chick Sox3 and Sox2 by Mouse Nodes of Different Genotype

<table>
<thead>
<tr>
<th>gsc genotype</th>
<th>Mouse node stage</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ES–MS</td>
<td>LS–0B</td>
</tr>
<tr>
<td>Sox3</td>
<td>8/9</td>
<td>10/12</td>
</tr>
<tr>
<td>+/+</td>
<td>0/1</td>
<td>16/19</td>
</tr>
<tr>
<td>+/−</td>
<td>1/4</td>
<td>8/19</td>
</tr>
<tr>
<td>Sox2</td>
<td>5/10</td>
<td>5/13</td>
</tr>
<tr>
<td>+/+</td>
<td>1/5</td>
<td>3/15</td>
</tr>
<tr>
<td>−/−</td>
<td>0/3</td>
<td>2/6</td>
</tr>
</tbody>
</table>

Note. All mice are β-gal⁺/⁺; the genotype for goosecoid is shown. Stages (Downs and Davies, 1993) of donor mouse embryos: ES, early streak; MS, mid-streak; LS, late streak; 0B, no allantoic bud. All chick hosts were at stage 3+/4 (Hamburger and Hamilton, 1951). Statistical analysis (χ², 2 × 2 contingency test, compared to wild type): *P < 0.002; **P < 0.02; ***P < 0.05.

The chick area opaca assay requires stronger inducing activity than does the prospective neural plate of the normal embryo and is therefore able to uncover more subtle consequences of the mutation. Whichever of these interpretations turns out to be correct, our results do reveal that goosecoid activity is required in the mouse node for its full inducing strength and that the phenotypic consequences of the lack of just one copy can be detected with this assay.

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REFERENCES


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