goosecoid and cerberus-like do not interact during mouse embryogenesis

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ABSTRACT Mouse Cerberus-like (Cer-l) is a neural inducer molecule, capable of inhibiting Nodal and BMP-4 signals in the extracellular space. The cer-l expression domain in the Anterior Visceral Endoderm (AVE) and prechordal plate, tissues involved in head induction and patterning, respectively, suggested a role for this gene in head formation. However, animals homozygous for the cer-l null allele failed to show any abnormality, leading us to propose the existence of other factor(s) that might compensate for cer-l loss-of-function. Since goosecoid (gsc) shares some domains of expression with cer-l and was shown to be essential for head morphogenesis, we tested its ability to interact genetically with cer-l. With this aim we generated cer-l/gsc double mutants. These animals were analyzed at birth for skeletal defects and revealed the same phenotype as gsc−/− single mutants. We also investigated the proper patterning of structures adjacent to the prechordal plate by performing in situ hybridization of HNF-3β, Six-3 and BF-1, genes whose expression domains remained unchanged. In conclusion, the analysis carried out indicated that gsc does not compensate for cer-l loss-of-function and that these genes do not interact genetically.

KEY WORDS: mouse cerberus-like, goosecoid, BMP-4, head induction, double mutants

Mouse cerberus-like (cer-l) encodes a secreted protein which binds to BMP-4 and Nodal in the extracellular space, thus preventing the binding of these ligands to their corresponding receptors (Belo et al., 2000). Its Xenopus counterpart, Cerberus (Xcer) was also found to be a XWnt-8 inhibitor (Piccolo et al., 1999), which cer-l was not (Belo et al., 2000). cer-l is expressed in the mouse embryo in tissues that are involved in head induction and patterning. At 5.5 d.p.c. cer-l is present in the Anterior Visceral Endoderm (AVE), the head organizing tissue; and at 7.5 d.p.c. cer-l transcripts can be found in the anterior endoderm and mesoderm of the prechordal and notochordal plates (Belo et al., 1997). Furthermore, in Xenopus animal cap experiments, cer-l mRNA, like Xcer, was shown to be a neural inducer: both mRNAs induce the expression of the pan-neural marker NCAM and the anterior neural marker otx2 (Bouwmeester et al., 1996; Belo et al., 1997).

All of these results, together with the fact that the related Xcer mRNA is capable of inducing an ectopic head when injected in the most ventral vegetal blastomere of the Xenopus embryo (Bouwmeester et al., 1996), suggested the involvement of cer-l in the mechanism of head induction in the mouse.

cer-l was inactivated by homologous recombination in ES cells and the resulting null mutants failed to show any defect (Belo et al., 2000; Stanley et al., 2000; Shawlot et al., 2000). This fact led to the proposal that another factor may compensate for the loss of function of cer-l. So far, no gene related to cer-l has been described in the mouse, thus, the mechanism of redundancy may rely on a molecule with functional similarities. In order to test for the compensation of cer-l by another BMP-4 inhibitor, we tested the interaction between noggin and cer-l by generating double mutants. These animals were analyzed and did not display any further defects besides the noggin phenotype, suggesting that noggin is not the factor that compensates for the loss of cer-l (Borges et al., 2001).

Here we report a similar study with another candidate gene, goosecoid (gsc). This homeobox containing gene, in Xenopus, represses the expression of BMP-4 in the marginal zone (Fainsod et al., 1994) and can induce the expression of chordin (Sasai et al., 1994), another BMP antagonist (Piccolo et al., 1996). In the mouse, gsc is expressed at various phases of embryogenesis (Blum et al., 1992). Before gastrulation gsc is expressed in the AVE (Belo et al., 1998) whereas during gastrulation it is expressed in regions of the

Abbreviations used in this paper: AVE, Anterior Visceral Endoderm; BMP-4, Bone Morphogenetic Protein-4; cer-l, cerberus-like; d.p.c., days postcoitum; gsc, goosecoid; mdkk1, mouse dickkopf-1; Xcer, Xenopus cerberus; XWnt8, Xenopus Wnt8.

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0214-6282/2002/$25.00
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Printed in Spain
www.ijdb.ehu.es
embryo with axial patterning activity – the anterior primitive streak, the node and the prechordal plate (Blum et al., 1992; Belo et al., 1998). At later stages of embryogenesis, at E10.5, gsc is expressed in craniofacial regions, ventral body wall and limbs (Gaunt et al., 1993; Belo et al., 1998). Homozygous gsc mutants generated by targeted inactivation in ES cells do not present any gastrulation defects, which would be related with its early phases of expression (Yamada et al., 1995; Rivera-Perez et al., 1995). However, the null mutants are lethal at birth due to craniofacial malformations, defects related with gsc expression during later phases of embryogenesis. Later on, these mutants were the subject of a detailed analysis at the level of the base of the skull and malformations correlated with the site of expression between 7.5 and 8.5 d.p.c. were described (Belo et al., 1998). These malformations are deletions and fusions in the midline of the prechordal chondrocranium, a region that develops in close association with the prechordal plate (where gsc is expressed), thus suggesting a role for gsc during gastrulation. In addition, in the study of the genetic interaction between gsc and HNF-3β, gsc was shown to play a role in axial development. The generation of gsc−/−;HNF3β−/− mutants led to the proposal that these genes act synergistically to regulate neural tube patterning and head development (Filosa et al., 1997). Double mutant studies have been very useful in the study of gene networks. For example, the chordin/noggin double mutant revealed the requirement of both BMP-4 antagonists emanating from the node in order to maintain the head inductive activity of the AVE (Bachiller et al., 2000).

Since gsc and cer-l share some domains of expression in the AVE and prechordal plate, and seem to be involved in the process of head formation, we decided to investigate the existence of functional redundancy between them. With this purpose, we generated cer-l−/−;gsc−/− double mutants using the same approach as described in Borges et al. (2001). Double heterozygous animals were intercrossed and a total of 84 neonates were recovered at birth and genotyped by PCR (Table 1). By analyzing the results of this genotyping, we could observe that all classes of genotypes are present at approximately the expected Mendelian ratio. Within the litters recovered, 19 animals died at birth and, from the observation of the external morphology, we did not detect any differences between them. After genotyping, we could observe that these newborns belonged to three different classes: cer-l+/−;gsc−/−, cer-l−/−;gsc+/- and cer-l−/−;gsc−/−, meaning that the lethality affects all gsc−/− classes, independently of the cer-l genotype. In order to study the recovered animals in detail and detect possible skeletal defects in addition to the ones described for gsc−/− single mutants, we performed Alcian Blue/Alizarin Red staining and carefully analyzed the base of the skull. Two classes of phenotypes were observed: the first corresponding to the wild type and the second composed of preparations that displayed the gsc−/− phenotype described by previous reports (Yamada et al., 1995; Rivera-Perez et al., 1995; Belo et al., 1998). These defects are visible in a dorsal view of the base of the cranium and consist in the loss of the tympanic rings, fusion of the ethmoid and the presphenoid into a single unit, and reduction of the vomer and the presphenoid (Fig. 1). These malformations are due to the proximity of the ancestral structure from which the prechordal cranium develops, the trabecula, and the prechordal plate, during skull morphogenesis (Belo et al., 1998). All the preparations that presented gsc null mutant phenotype belonged to the following classes of genotypes: cer-l+/−;gsc−/−, cer-l−/−;gsc+/- and cer-l−/−;gsc−/−. As we did not observe increased severity of the defects in the double mutant neonates when compared with gsc−/− single mutants, we went on to study the existence of abnormalities during earlier stages of development. With this purpose, we dissected 44 embryos at 9.5 d.p.c. and genotyped them by PCR. The results of genotyping are

### Table 1

**RESULTS OF GENOTYPING OF NEONATES RECOVERED FROM CER-L−/−; GSC−/− INTERCROSSES**

<table>
<thead>
<tr>
<th></th>
<th>Gsc−/−Cer−/−</th>
<th>Gsc−/−Cer−/−</th>
<th>Gsc−/−Cer−/−</th>
<th>Gsc−/−Cer−/−</th>
<th>Gsc−/−Cer−/−</th>
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<td>10.50</td>
<td>5.25</td>
<td>10.50</td>
<td>5.25</td>
</tr>
<tr>
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<td>13.10%</td>
<td>7.14%</td>
<td>20.24%</td>
<td>15.48%</td>
<td>5.95%</td>
<td>13.10%</td>
<td>3.57%</td>
</tr>
<tr>
<td>% Observed</td>
<td>4.76%</td>
<td>16.67%</td>
<td>13.10%</td>
<td>7.14%</td>
<td>20.24%</td>
<td>15.48%</td>
<td>5.95%</td>
<td>13.10%</td>
<td>3.57%</td>
</tr>
</tbody>
</table>

N=number of neonates recovered

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**Fig. 1.** cer-l−/−;gsc−/− neonates display the same defects presented by gsc−/− single mutants. (A) Wild type neonate. (B) cer-l−/−;gsc−/− littermate does not present defects. (C) cer-l−/−;gsc−/− show reduction of the vomer (V), fusion of the ethmoid (Eth) with the presphenoid (PS; white arrow) and absence of the tympanic rings (black arrowhead). (D) cer-l−/−;gsc−/− littermate presents the wild type phenotype. (E) cer-l−/−;gsc−/− and (F) cer-l−/−;gsc−/− present the same defects as cer−/−;gsc−/− (compare with C). The skeletons of neonatal mice were stained with alcian blue (for cartilage) and alizarin red (for bone). BO, basioccipital; BS, basisphenoid; O, occipital.
resumed in Table 2. All the classes of genotypes are present, and, as in the case of the neonates, at approximately the expected Mendelian percentages.

To study the role of these genes in the prechordal plate, we decided to investigate the existence of abnormalities in the structures that develop adjacent do it. For that purpose, we performed mRNA in situ hybridization for the forebrain markers Six-3 and BF-1 and for the axial marker HNF-3β. Six-3 is normally expressed in the telencephalon, the ventral diencephalon, the developing eye and the Rathke’s pouch (Oliver et al., 1995). Since it has been suggested that the prechordal plate may be involved in the patterning of the forebrain, we expected that the normal expression of this marker would be affected in the double mutants. Figure 2 A-C displays Six-3 expression in 9.5 d.p.c. embryos. By comparison between the wild type (Fig. 2A) and the mutant embryos (Fig. 2 B,C), we can observe that Six-3 expression domain is unaltered in either the single gsc−/− mutant or in the cer-l−/−gsc−/− compound mutant. The results of BF-1 in situ hybridization confirm the lack of abnormalities in forebrain patterning of the studied mutants. As we can observe in the wild type 9.5 d.p.c. embryo (Fig. 2D), BF-1 is expressed in the telencephalon (Tao and Lai, 1992). In the mutants (Fig. 2 E,F), the expression of this marker is unaltered in both gsc−/− mutants and cer-l−/−gsc−/− double mutants. We have also analyzed the expression of HNF-3β mRNA in 9.5 d.p.c. embryos. At this stage, this gene is normally expressed along the anterior-posterior (A-P) axis in the notochord, the neural tube and floorplate, with its anterior limit at the level of the posterior diencephalon (Filosa et al., 1997). The rostral limit of this expression domain is adjacent to the prechordal plate, so, we considered the hypothesis of an abnormal HNF-3β expression pattern in the region associated with the prechordal plate. However, by the observation of the results of the in situ hybridization (Fig. 2 G-I), we could see that the pattern of expression of HNF-3β is also unaffected in both classes of mutants (Fig. 2 H, I). The expression in the notochord and floorplate is normal along the A-P axis of these embryos.

Taken together, our results indicate that the double mutants cer-l−/−;gsc−/−, do not display patterning defects neither at the level of the forebrain nor in the midline tissues along the body axis. At the level of the skull, the defects visible in the cer-l−/−;gsc−/− mutant coincide with the ones present in the gsc−/− single mutant. These results led us to conclude that cer-l and gsc do not interact genetically during mouse embryogenesis and that gsc is not the factor that compensates for the loss of function of cer-l.

In vertebrates, in addition to gsc, two goosecoid related genes were described, gxs, in chick (Lemaire et al., 1997) and gsc-like (gscl) in the mouse (Galli et al., 1997). gxs has not been cloned in the mouse, but sequence comparisons strongly indicate that gxs and gscl represent distinct genes in amniotes (Belo et al., 1998), suggesting that gsc may be redundant with these gsc-related genes. Despite of these genes being expressed in the prechordal plate, their expression pattern has not been described in the AVE, nor in the topological equivalent tissue in the chick, the hypoblast; and they have not been implicated in head formation/morphogenesis, therefore, they are unlikely to compensate for cer-l loss of function.

It has been proposed that trunk signals, like Nodal, Wnts and BMPs, must be inhibited in order to allow for the induction of the anterior head field. (Piccolo et al., 1999). According to this model, the role of the AVE is to secrete molecules that inhibit the posteriorizing action of factors such as Nodal, Wnts and BMPs. Molecules secreted by the AVE that may play this role are the nodal antagonist, Lefty-1, the Wnt inhibitor, Dickkopf-1 (Mdkk1) and Cer-

**TABLE 2**

<table>
<thead>
<tr>
<th>Gsc+/Cer+/</th>
<th>Gsc+/Cer-</th>
<th>Gsc-/Cer+</th>
<th>Gsc-/Cer-</th>
<th>Gsc+/-Cer+</th>
<th>Gsc+/-Cer-</th>
<th>Gsc-/Cer+</th>
<th>Gsc-/Cer-</th>
<th>Gsc+/-Cer+</th>
<th>Gsc+/-Cer-</th>
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</thead>
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<td>4.00</td>
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<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
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<tr>
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<td>5.00</td>
<td>2.75</td>
</tr>
<tr>
<td>% Observed</td>
<td>2.27%</td>
<td>27.27%</td>
<td>9.09%</td>
<td>9.09%</td>
<td>27.27%</td>
<td>4.55%</td>
<td>6.82%</td>
<td>6.82%</td>
<td>6.82%</td>
</tr>
<tr>
<td>% Expected</td>
<td>6.25%</td>
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<td>6.25%</td>
<td>12.50%</td>
<td>25.00%</td>
<td>12.50%</td>
<td>6.25%</td>
<td>12.50%</td>
<td>6.25%</td>
</tr>
</tbody>
</table>

N=number of embryos recovered
I. By inhibiting these signals, the AVE and the underlying anterior epiblast become regions free of posteriorizing agents, thus, allowing the formation of the anterior head (Piccolo et al., 1999).

Since cer-l is not compensated by gsc, neither by noggin (Borges et al., 2001) nor chordin (E. M. De Robertis, personal communication), we propose that it may interact with other genes expressed in the AVE. Data from several studies have suggested an interaction between cer-l and otx-2. In Xenopus animal cap assays, cer-l induces otx-2 expression (Belo et al., 1997) and experiments of tissue recombination in the mouse, also reveal the requirement of cer-l for the maintenance of otx-2 expression (Shawlot et al., 2000). Taken together, these observations point to the existence of some interaction between cer-l and otx-2. The generation of the cer-l;otx-2 double mutant may help to unravel this process. Other antagonists expressed in the AVE that may play key roles in the restriction of posteriorizing factors, like lefty-1 and mdkk-1, may be redundant with cer-l. Therefore, the generation of further cer-l;double mutants may contribute to a better understanding of the mechanisms of early mouse patterning and head induction.

**Experimental Procedures**

**Generation and Genotyping of Double Mutants**

cer-l -/- heterozygous mice were intercrossed with gsc+/- heterozygous mice (both of C57/B6 background), originating double heterozygous animals as described in Bachiller et al. (2000). Genotyping of cer-l and gsc was determined by PCR as described in Belo et al. (2000) and Belo et al. (1998), respectively.

**Skeletal Analysis and In Situ Hybridization**

For the skeletal analysis of the neonates, Alcian Blue/Alizarin Red staining was performed as described in Belo et al. (1998). Whole mount in situ hybridization and anti-sense probe preparation was carried out as described in Belo et al. (1997). The plasmids containing Six-3, BF-1 and HNF-3β fragments were cut with XbaI, BamH1 and Asp700, respectively, and transcribed using T7 RNA polymerase.

**Acknowledgements**

We thank E. M. De Robertis for the gift of the goosecoid mutant mice and for his encouragement and continuous support. A. C. Borges and S. Marques are recipients of F.C.T. fellowships. This work was supported by a research grant of the Fundaçao Calouste Gulbenkian/Instituto Gulbenkian de Ciência to J. A. Belo of which he is a member researcher.

**References**


Received: September 2001
Reviewed by Referees: November 2001

Modified by Authors and Accepted for Publication: January 2002