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Short Communication

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 extracelular 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 dickhopf-1; *Xcer*, Xenopus cerberus; *X*Wnt8, *Xenopus* Wnt8.

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TABLE 1

RESULTS OF GENOTYPING OF NEONATES RECOVERED FROM *CER-L*$^{+/-}$; *GSC*$^{+/-}$ INTERCROSSES

	Gsc$^{+/+}$Cer$^{+/+}$	Gsc$^{+/+}$Cer$^{+/-}$	Gsc$^{+/+}$Cer$^{-/-}$	Gsc$^{+/-}$Cer$^{+/+}$	Gsc$^{+/-}$Cer$^{+/-}$	Gsc$^{+/-}$Cer$^{-/-}$	Gsc$^{-/-}$Cer$^{+/+}$	Gsc$^{-/-}$Cer$^{+/-}$	Gsc$^{-/-}$Cer$^{-/-}$	N
No. Observed	4.00	14.00	11.00	6.00	17.00	13.00	5.00	11.00	3.00	84.00
No. Expected	5.25	10.50	5.25	10.50	21.00	10.50	5.25	10.50	5.25	84.00
%Observed	4.76%	16.67%	13.10%	7.14%	20.24%	15.48%	5.95%	13.10%	3.57%	100%
%Expected	6.25%	12.50%	6.25%	12.50%	25.00%	12.50% 1	6.25%	12.50%	6.25%	100%

N=number of neonates recovered

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

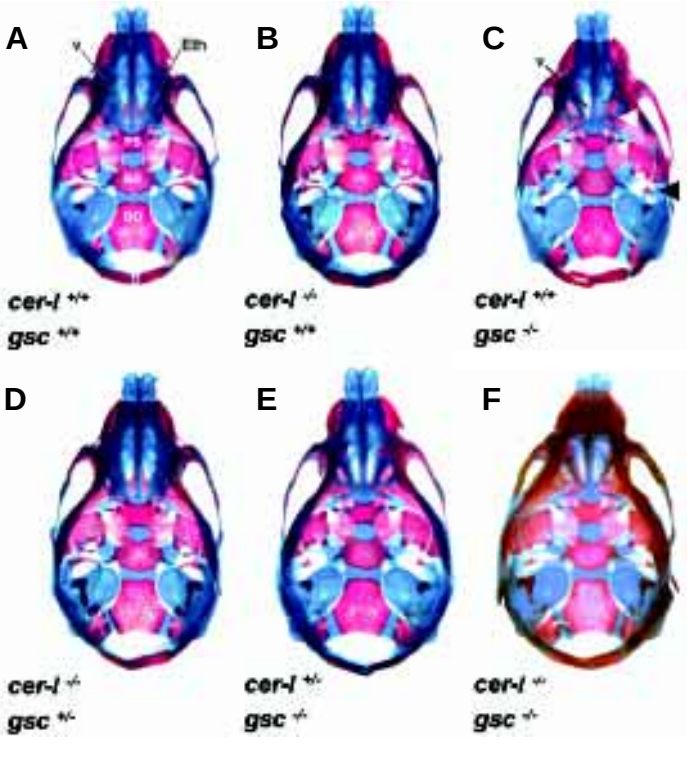

Fig. 1. *cer-l-/-;gsc-/-* neonates display the same defects presented by *gsc-/-* single mutants. (A) *Wild type neonate.* **(B)** cer-l$^{-/-}$;gsc$^{+/+}$ *littermate does not present defects.* **(C)** cer-l$^{+/+}$;gsc$^{-/-}$ *show reduction of the vomer (V), fusion of the ethmoid (Eth) with the 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-l$^{+/+}$;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.*

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*$^{-/-}$*;HNF3*β$^{+/-}$ 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 usefull in the study of gene netwoks. 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 Mendellian 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

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 could expect 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 unchanged 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 analized 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, *gsx*, in chick (Lemaire *et al.*, 1997) and *gsc-like (gscl)* in the mouse (Galili *et al.*, 1997). *gsx* has not been cloned in the mouse, but sequence comparisons strongly indicate that *gsx* 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.

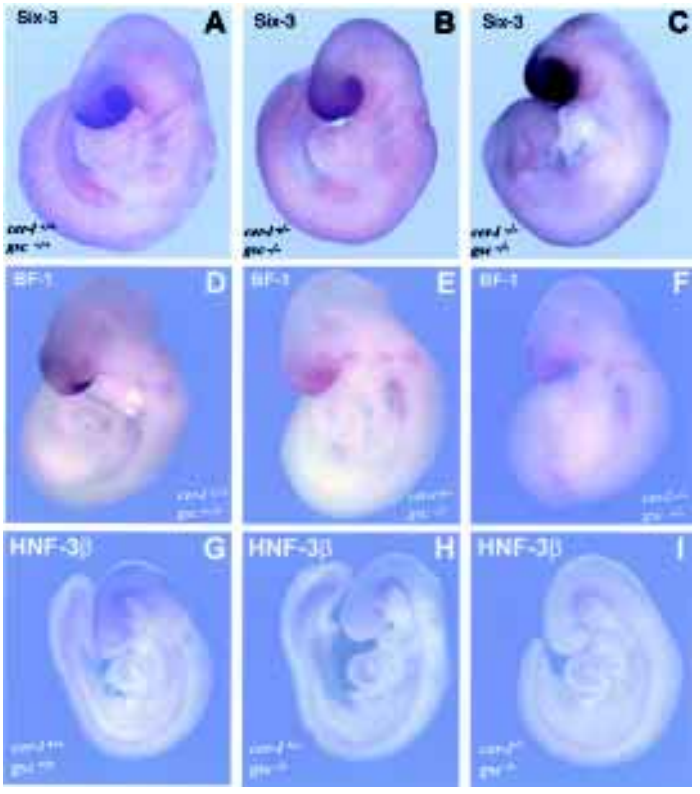

Fig. 2. *Six-3, BF-1* and *HNF-3β* mRNA *in situ* hybridization in 9.5 d.p.c. embryos. (A) *Wild type embryo showing* Six-3 *expression in the telencephalon, the ventral diencephalon and the developing eye.* **(B)** cer-l$^{+/-}$;gsc$^{-/-}$ *and* **(C)** cer-l$^{-/-}$;gsc$^{-/-}$ *mutant embryos exhibiting an unaltered* Six-3 *expression pattern.* **(D)** *Wild type embryo presenting* BF-1 *expression in the telencephalon.* **(E)** cer-l$^{+/-}$;gsc$^{-/-}$ *and* **(F)** cer-l$^{-/-}$;gsc$^{-/-}$ *embryos do not present any alteration in* BF-1 *expression relative to the wild type littermate.* **(G)** *Wild type embryo showing* HNF-3β *expression along the notochord, neural tube and floorplate.* **(H)** cer-l$^{+/-}$;gsc$^{-/-}$ *and* **(I)** cer-l$^{-/-}$;gsc$^{-/-}$ *mutant embryos present the same* HNF-3β *expression pattern as shown in (G). The tail of the embryo in (I) was removed for photographic purposes.*

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, Dickhopf-1 (Mdkk1) and Cer-

TABLE 2

RESULTS OF GENOTYPING OF EMBRYOS RECOVERED FROM *CER-L*$^{+/-}$*; GSC*$^{+/-}$ INTERCROSSES

	Gsc$^{+/+}$Cer$^{+/+}$	Gsc$^{+/+}$Cer$^{+/-}$	Gsc$^{+/+}$Cer$^{-/-}$	Gsc$^{+/-}$Cer$^{+/+}$	Gsc$^{+/-}$Cer$^{+/-}$	Gsc$^{+/-}$Cer$^{-/-}$	Gsc$^{-/-}$Cer$^{+/+}$	Gsc$^{-/-}$Cer$^{+/-}$	Gsc$^{-/-}$Cer$^{-/-}$	N
No. Observed	1.00	12.00	4.00	4.00	12.00	2.00	3.00	3.00	3.00	44.00
No. Expected	2.75	5.50	2.75	5.50	11.00	5.50	2.75	5.50	2.75	44.00
% Observed	2.27%	27.27%	9.09%	9.09%	27.27%	4.55%	6.82%	6.82%	6.82%	100%
% Expected	6.25%	12.50%	6.25%	12.50%	25.00%	12.50%	6.25%	12.50%	6.25%	100%

N=number of embryos recovered

l. 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 *cerl-*. 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 that were intercrossed to obtain double mutants. For genotyping, DNA was prepared from tail biopsies of adult and newborn mice and from the extraembryonic membranes of 9.0 and 9.5 d.p.c. embryos, 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.

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