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Short Communication

The BMP antagonists cerberus-like and noggin do not interact during mouse forebrain development

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ABSTRACT Mouse *cerberus-like* encodes for a secreted factor of the Cerberus/Dan family. This molecule has neural inducing capabilities and can bind to BMP-4 and nodal molecules in the extracellular space. When *cerberus-like* is inactivated, its function may be compensated for another molecule, since no abnormalities can be observed in the mouse mutant. Compensation mechanisms have been shown to occur between the BMP antagonists chordin and noggin. Here we report the generation of *cerberus-like*$^{-/-}$;*noggin*$^{-/-}$ double mutants to uncover a possible compensation by *noggin* in *cer-l*$^{-/-}$ mutant. Double mutants were obtained and failed to show any further detectable defects beside the ones presented by the *noggin*$^{-/-}$ single mutant. Contrarily to *chordin* and *noggin*, mouse *cerberus-like* and *noggin* cannot compensate for each other during mouse embryogenesis.

KEY WORDS: *mouse cerberus-like; noggin; BMP-4; head induction*

The secreted factors chordin, noggin, follistatin and cerberus (Reviewed in De Robertis *et al.,* 1997) share a common biochemical activity: BMP-4 (bone morphogenetic protein-4) inhibition by direct binding to it (Iemura *et al.,* 1995; Piccolo *et al.,* 1996; Zimmerman *et al.,* 1996; Piccolo *et al.,* 1999). This antagonism leads to a graded inhibition of ventral BMP signaling and is essential for neural induction and dorsoventral patterning of the vertebrate embryo (De Robertis and Sasai, 1996).

Xenopus cerberus (*Xcer*) encodes for a secreted protein with neural inducing activity (Bouwmeester *et al.,* 1996). Besides having anti-BMP-4 activity, XCer was shown to inhibit Xnr-1 (Xenopus nodal related-1) and Xwnt-8 by direct binding to them (Piccolo *et al.,* 1999). In the mouse embryo, a gene related to *Xenopus cerberus*, mouse *cerberus-like* (m*cer-l*) was isolated (Belo *et al.,* 1997; Biben *et al.,* 1998; Shawlot *et al.,* 1998). This gene starts to be expressed at 5.5 days postcoitum (d.p.c.) in the Anterior Visceral Endoderm (AVE). At neural plate stage, the expression is found in the endoderm underlying anterior neural plate. In histological sections, expression is found in all cells of the midline from the rostral end of the embryo to the proximity of the node, and includes anterior endoderm and mesoderm from the prechordal and notochordal plates (Belo *et al.,* 1997). Animal cap explants experiments led to the conclusion that both *Xcer* and m*cer-l* can induce the same molecular markers like *Otx2*, although *mcer-l* mRNA was not able to induce ectopic head-like structures as in the case of *Xcer* mRNA injection. Later, it was demonstrated that mCer-*l* exhibits anti-BMP as well as anti-Nodal activities, but unlike *Xenopus* Cerberus cannot inhibit XWnt8 signaling. This activity together with its expression in the AVE, a tissue implicated in the induction of the forebrain, suggested that mCer-l could be an important factor for head development (Belo *et al.,* 1997). However, careful phenotypical analysis of induced *cer-l*$^{-/-}$ loss of function animals failed to show any defect (Belo *et al.* 2000; Stanley *et al.,* 2000; Shawlot *et al.,* 2000).

This result suggests that some genes may be compensating for the lack of function of *cer-l*. This phenomenon was shown to occur between the BMP inhibitors Chordin and Noggin, as described in Bachiller *et al.* (2000). Targeted inactivation of *noggin* originates a recessive lethal phenotype during embryogenesis. The mutants display a vast number of defects in spinal cord and somites (McMahon *et al.,* 1998; Brunet *et al.,* 1998). *Chordin* null mutation results in stillborn animals which show abnormalities in ear development, pharyngeal and cardiovascular organization (Bachiller *et al.,* manuscript in preparation). Both genes are expressed in the node of the mouse embryo, at late gastrula stage. Later they are co-expressed at the level of the notochordal and prechordal plates. The double homozygous *chordin;noggin* mutants present defects at the level of the forebrain development in addition to defects related to left-right patterning and mesoderm maintenance (Bachiller *et al.,* 2000). In that study the double mutant shows defects not exhibited by the single mutations alone, meaning that some compensation was occurring when only one BMP inhibitor was missing.

This fact led us to test whether *cer-l* and *noggin* could compensate for the lack of function of each other. Although *cer-l* has other activities, they are both BMP-4 inhibitors sharing some domains of

Abbreviations used in this paper: AVE, Anterior Visceral Endoderm; BMP, Bone Morphogenetic Protein; CNS, Central Nervous System; d.p.c., days postcoitum; En-1, Engrailed-1; m*cer-l*, mouse *cerberus-like;* nog, noggin; Shh, Sonic hedgehog; *X*Cer, *Xenopus* Cerberus; *X*nr-1, *Xenopus* nodal-related-1; *X*Wnt8, *Xenopus* Wnt8;

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expression at the level of the prechordal and notochordal plates. In the present work, we used a similar approach as in Bachiller *et al.*(2000) for the generation of *cer-l*;*noggin* double mutants.

For that purpose, 160 neonates resultant from intercrosses of compound heterozygous mice (*nog*$^{+/-}$;*cer-l*$^{+/-}$) were recovered. Among them, 5 resembling noggin mutants (McMahon et al. 1998) were born dead, presented open neural tube, shortened body axis, truncated limbs, no tail and variability in cranial neural tube closure. After genotyping the neonates by PCR (Fig. 1), it was found that all possible genotype combinations were observed (Table 1) but not in accordance with the expected Mendelian rate: the combinations *nog*$^{-/-}$;*cer-l*$^{+/+}$, *nog*$^{-/-}$;*cer-l*$^{+/-}$ and *nog*$^{-/-}$;*cer-l*$^{-/-}$ accounted for only 5 newborns while a total of 40 were expected, a sign of embryonic lethality. Contrarily to all other genotypes, these combinations display the defects described above. The common feature of these three genotypes is the noggin homozygous mutation. All *nog*$^{-/-}$ neonates, independently of the *cer-l* genotype display severe abnormalities, the same ones as described for *noggin* null mutation (McMahon *et al.* 1998).

In contrast, *the cer-l* null mutant neonates do not show any defects (as reported in Belo *et al.,* 2000*;* Stanley *et al.,* 2000; Shawlot *et al.,* 2000). *nog*$^{+/-}$;*cer-l*$^{-/-}$, a genotype that could lead to defects related to a dose dependent mechanism, are also normal, at least by observation of the external morphology. Curiously, the double mutants *nog*$^{-/-}$;*cer-l*$^{-/-}$ show the same set of abnormalities as the *noggin*$^{-/-}$ mutant alone.

We decided to assess for morphological defects at the base of the cranium by Alcian blue/ Alizarin red staining. The skulls were dissected to allow the careful observation of its base (Fig. 2). Again, defects were observed only in *noggin* mutants, independently of the *cer-l* genotype (compare Figs. 2 A,B,C with 2 D,E,F). Due to *nog*$^{-/-}$ mutation, skeletal preparations are fragile and some bones may be broken during the procedure. In these preparations we can observe the fusion of presphenoid, basisphenoid and basioccipital bones (Fig. 2 D,E,F). This can readily be seen because frontal, parietal and interparietal bones are loose or absent. In the cases where these bones were absent, the neonates had the brain exposed at birth. In addition, occipital condyles are also loose, showing variability in the distance separating them. In cases of a large separation between the two occipital condyles, the occipital and basioccipital bones might not remain in place during bone/cartilage staining procedure, leading to differences in the preparations obtained (Fig. 2F). Nevertheless, these differences are caused by an artifact rather than a defect in the occipital and basioccipital bones. These bones were always present and were not affected in abnormal animals. This abnormal phenotype at the base of the cranium was observed in *nog*$^{-/-}$;*cer-l*$^{+/+}$, *nog*$^{-/-}$;*cer-l*$^{+/-}$ and *nog*$^{-/-}$;*cer-l*$^{-/-}$, meaning that the additional loss-of-function of *mcer-l* does not cause any abnormality besides the ones presented by noggin single mutants.

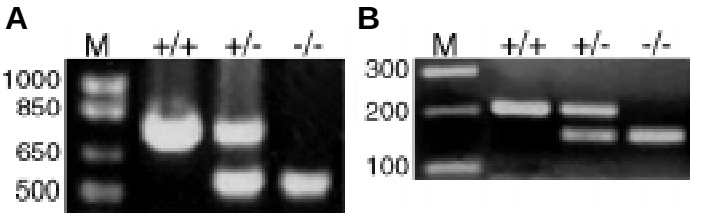

Fig. 1. PCR analysis of intercrosses. (A) *Genotyping for cerberus-like wild type and mutated alleles.* **(B)** *Noggin genotyping. The molecular marker (M) is on the left lane; the numbers indicated correspond to base pairs (bp).*

We then, dissected pregnant mothers at 9.5 and 10.5 d.p.c. to assess for defects visible only in earlier stages of development. The yolk sac was used for genotyping these embryos. Embryo genotyping results are resumed in Table 2. By these results one can see that all genotype combinations are now present approximately at the expected Mendelian ratio. We found 8 empty deciduas meaning that some embryos are reabsorbed during development due to mortality. Once again, all noggin mutants (regardless of *cer-l* genotype) show abnormalities in neu-

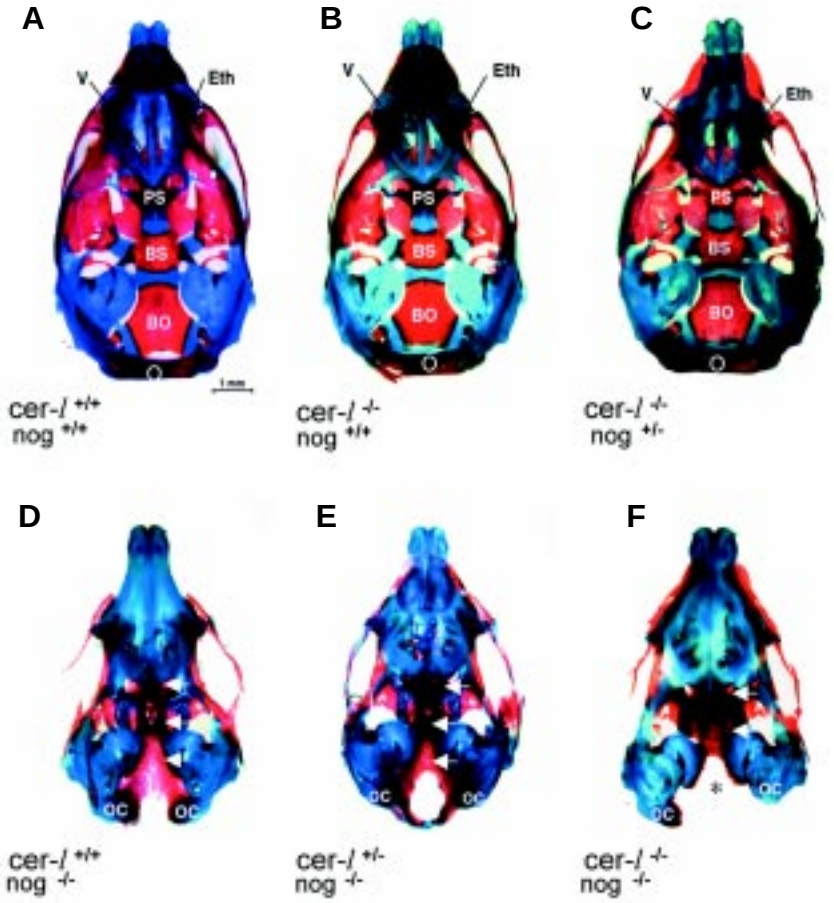

Fig. 2. Cer-l$^{-/-}$**;nog**$^{-/-}$ **neonates show bone fusions in the base of the cranium, the same defect presented by nog**$^{-/-}$ **single mutants. Dorsal views of the base of the cranium. (A)** *Wild type neonate.* **(B)** *Cer-l*$^{-/-}$*;nog*$^{+/+}$ *and* **(C)** *Cer-l*$^{-/-}$*;nog*$^{+/-}$ *littermates do not present defects.* **(D)** *Cer-l* $^{+/+}$*;nog*$^{-/-}$ *shows fusion of the presphenoid (PS), basisphenoid (BS) and basioccipital (BO) forming a single unit (arrows) and absence of cartilage between these bones.* **(E)** *Cer-l* $^{+/-}$*;nog*$^{-/-}$ *displays the same set of defects as the ones observed in Cer-l*$^{+/+}$*;nog*$^{-/-}$. **(F)** *Cer-l*$^{-/-}$*;nog*$^{-/-}$ *double mutant exhibits the same abnormalities as in* **(D)** *and* **(E)**. *Absence of BO (*) is the result of degradation of the tissue during preparation of the specimen. The removal of cer-l and noggin does not alter the phenotype observed in the nog single mutant (compare with panels D and E). The skeletons of neonatal mice were stained with alcian blue and alizarin red for cartilage and bone, respectively. BO, basioccipital; BS, basisphenoid; Eth, ethmoid; O, occipital; OC, occipital condyles; PS, presphenoid; V, vomer.*

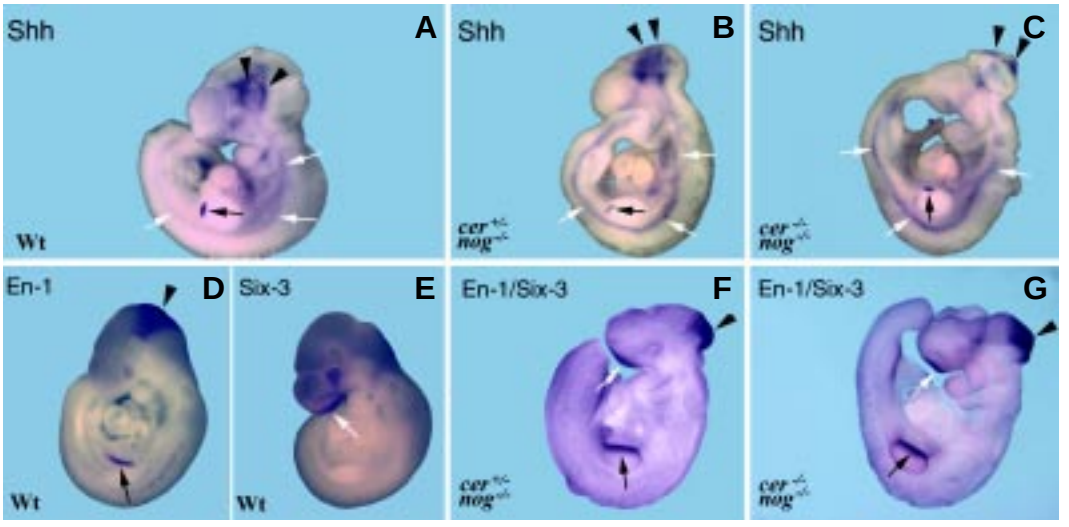

Fig. 3. mRNA *in situ* hybridization in 10.5 d.p.c. embryos. (A) *Wild type embryo showing Shh expression in the ventral fore- midbrain (black arrowheads), ventral neural tube (white arrows) and forelimb ZPA (black arrow).* **(B,C)** *Mutant embryos exhibit an unaltered Shh expression pattern, in spite of having an open neural tube in the brain (arrowheads) and anterior spinal cord.* **(D)** *Wild type embryo showing En-1 expression in the mid-hindbrain boundary (black arrowhead) and in the limb bud (black arrow).* **(E)** *Expression of Six-3 in the ventral forebrain (white arrow) in a wild type embryo.* **(F,G)** *Expression of En-1 and Six-3 shows that in the mutants, brain regionalization is not affected as judged by their expression in the forebrain (white arrows) and in the mid-hindbrain boundary (black arrowheads). En-1 expression in the limb buds (black arrows) is also unaffected.*

ral tube closure, particularly in the brain vesicles between diencephalon and myelencephalon, but sometimes from the diencephalon to the caudal limit of the embryo.

In order to uncover the existence of dorsoventral patterning defects caused by excessive BMP-4 activity, we performed *sonic hedgehog (Shh)* mRNA *in situ* hybridization in embryos of 10.5 d.p.c. At this stage, *sonic hedgehog* is expressed in the ventral diencephalon, floor plate and notochord (Fig. 3). Expression is also detected in the zone of polarizing activity of the limb buds. In the noggin null mutant *shh* expression pattern is unaltered at the level of the central nervous system (CNS) and forelimbs but is severely reduced at hindlimb level, where no *shh* expression is detected at the ventral midline of the neural tube (McMahon *et al.,* 1998). Interestingly, the same result was obtained for double mutants *cer-l*$^{-/-}$;*nog*$^{-/-}$. Also, expression at the level of the CNS seemed unaltered in *cer-l*$^{-/-}$;*nog*$^{-/-}$ mutants (Fig. 3 B,C). *In situ* hybridization using *En-1*, a mid-hindbrain and limb bud marker (Joyner *et al.,* 1987) and *Six-3*, an anterior forebrain marker (Oliver *et al.,* 1995) as probes, also showed no alteration in the double mutant (Fig. 3 D-G).

Defects in the midline of the cranium could also be observed in *goosecoid-1* (*gsc-1*) null mutants as described by Belo *et al.* (1998). According to these authors, the midline defects of those mutants develop in close association with *goosecoid* expressing cells at the prechordal plate. The region where the defects appear (vomer, ethmoid and presphenoid bones) is previously occupied by the prechordal plate. In *nog*$^{-/-}$ the defective bones of the cranium are essentially the presphenoid, basisphenoid and basioccipital and the region where these bones are formed is previously occupied by notochord and prechordal plates, where *cer-l* expression can also be observed. The fact that these midline defects are observed in *nog*$^{-/-}$;*cer-l*$^{+/+}$, *nog*$^{-/-}$;*cer-l*$^{+/-}$ and *nog*$^{-/-}$; *cer-l*$^{-/-}$ and not in animals with the rest of the genotypes, means that the loss of function of the *cer-l* gene, when accumulated with the *noggin* mutation does not cause additional abnormalities. *Noggin* transcripts can be seen at early stages of development in these structures and probably, the bone malformations observed here are related to misregulation of BMP activity in the notochordal and prechordal plates.

In addition, *nog*$^{-/-}$ mutation causes bone malformations in the entire skeleton (Brunet *et al.,* 1998). Misregulation of the factors that control this mechanism, like BMPs, results in lack of definition of shape of each skeletal element. This happens in the cranium of *noggin* mutants, because some of the bones of the midline, did not acquire the correct shape becoming fused. However, the loss of function of *cer-l* in addition to *nog*$^{-/-}$ mutation was expected to

TABLE 1

GENOTYPING RESULTS OF THE NEONATES RECOVERED FROM *CER-l*$^{+/-}$;*NOG*$^{+/-}$ INTERCROSSES

	Nog$^{+/+}$Cer$^{+/+}$	Nog$^{+/+}$Cer$^{+/-}$	Nog$^{+/+}$Cer$^{-/-}$	Nog$^{+/-}$Cer$^{+/+}$	Nog$^{+/-}$Cer$^{+/-}$	Nog$^{+/-}$Cer$^{-/-}$	Nog$^{-/-}$Cer$^{+/+}$	Nog$^{-/-}$Cer$^{+/-}$	Nog$^{-/-}$Cer$^{-/-}$	N
No. Observed	19	21	14	29	45	27	1	2	2	160 No.
Expected	10	20	10	20	40	20	10	20	10	160 %
Observed	11.88%	13.13%	8.75%	18.13%	28.13%	16.88%	0.63%	1.25%	1.25%	100%
% Expected	6.25%	12.50%	6.25%	12.50%	25.00%	12.50%	6.25%	12.50%	6.25%	100%

N=number of neonates recovered

TABLE 2

GENOTYPING RESULTS OF THE EMBRYOS RECOVERED FROM *CER-l*$^{+/-}$;*NOG*$^{+/-}$ INTERCROSSES

	Nog$^{+/+}$Cer$^{+/+}$	Nog$^{+/+}$Cer$^{+/-}$	Nog$^{+/+}$Cer$^{-/-}$	Nog$^{+/-}$Cer$^{+/+}$	Nog$^{+/-}$Cer$^{+/-}$	Nog$^{+/-}$Cer$^{-/-}$	Nog$^{-/-}$Cer$^{+/+}$	Nog$^{-/-}$Cer$^{+/-}$	Nog$^{-/-}$Cer$^{-/-}$	N
No. Observed	1.00	5.00	8.00	5.00	14.00	11.00	1.00	8.00	4.00	57
No. Expected	3.56	7.13	3.56	7.13	14.25	7.13	3.56	7.13	3.56	57 %
Observed	1.75%	8.77%	14.04%	8.77%	24.56%	19.30%	1.75%	14.04%	7.02%	100%
% Expected	6.25%	12.50%	6.25%	12.50%	25.00%	12.50%	6.25%	12.50%	6.25%	100%

Number of empty deciduas: 8; N=number of embryos analysed

increase BMP activity. Subsequently, this double mutation could have led to a more severe phenotype than the ones observed in *nog*$^{-/-}$ single mutant, because the expression of both genes partially overlaps in the regions where the base of the cranium will form (prechordal and notochordal plates). However, this did not occur and unless some subtle abnormalities could have been missed in this analysis, cumulative defects in *cer-l*$^{-/-}$;*nog*$^{-/-}$ double mutants were not found.

This is further confirmed by the *sonic hedgehog in situ* hybridization. We used this probe to assess for dorsoventral patterning defects as BMPs have been implicated in this process. Since *noggin* and *mcer-l* are BMP antagonists, we adressed the relationship between the excessive BMP activity and the dorsoventral patterning of the CNS. *Shh* is responsible for inducing ventral cell fates and is expressed in the floor plate and dorsal side of the notochord. Failure in CNS dorsoventral patterning would lead to displacement of expression of *Shh*. In *nog*$^{-/-}$ single mutants *Shh* expression pattern is unaltered in the anterior region of the spinal cord and notochord. In accordance with our previous results the *Shh* expression pattern is identical in *nog*$^{-/-}$ single mutant and in *nog*$^{-/-}$;*cer-l*$^{-/-}$ double mutant.

In conclusion, the interaction between *cer-l* and *noggin* could have led to defects at various levels. The first being defects in D-V patterning; the second being composed of mesodermal defects because BMP-4 is essential for mouse gastrulation, for mesoderm formation and neural induction (Winnier *et al.,* 1995). Excess of BMP-4 activity, in this case provided by lack of BMP inhibitors, could lead to mesoderm and more importantly to neural defects during embryogenesis. This fact was not observed in *nog*$^{-/-}$;*cer-l*$^{-/-}$ embryos. Defects in the head could also have been expected because *mcer-l* is expressed in the AVE and prechordal plate, tissues implicated in head induction and patterning respectively. From our results we can conclude that *noggin* cannot compensate for the loss-of-function of *cer-l,* since the removal of the two gene products shows only the defects visible in *nog*$^{-/-}$ single mutants. The question that led us to test this interaction remains: which factors may be compensating for the *cer-l* loss of function? We propose that other BMP antagonists might be performing this function. Another possibility is the existence of other members of the Cerberus family that may compensate for lack of *cer-l* activity. Cerberus-like has also been shown to be a Nodal antagonist (Belo *et al.,* 2000).

To clarify these questions it would be of interest to generate other double mutant combinations: *chordin;cer-l* to test for the compensation of the anti BMP activity; *Otx2;cer-l* to test for the *cer-l* function in the AVE; *gsc-1;cer-l* to understand the function of *cer-l* in the prechordal plate and in the formation of the cranium; and *Lefty-1*;*cer-l* to test the compensation by other Nodal antagonist.

Experimental procedures

Generation and genotyping of double mutants

cer-l$^{+/-}$ heterozygous mice were crossed with *noggin*$^{+/-}$ heterozygous mice both of 129/Sv 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 9.5 and 10.5 d.p.c. embryos, as described by Bachiller *et al.* (2000). Genotyping of *cer-l* and *noggin* was determined by PCR as described in Belo *et al.* (2000) and McMahon *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 containig *Shh*, *En-1* and *Six-3* fragments were cut with *Hind*III, *Cla*I and *Xba*I, respectively. Shh probe was transcribed with T3 RNA polymerase; En-1 and Six-3 were both transcribed using T7 RNA polymerase.

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