Development 134, 2051-2060 (2007) doi:10.1242/dev.000901

Cerberus is a feedback inhibitor of Nodal asymmetric signaling in the chick embryo

Ana Teresa Tavares[1,*], Sofia Andrade[1], Ana Cristina Silva[1,2] and José António Belo[1,2,*]

The TGF-β-related molecule Nodal plays an essential and conserved role in left-right patterning of the vertebrate embryo. Previous reports have shown that the zebrafish and mouse Cerberus-related proteins Charon and Cerberus-like-2 (Cerl-2), respectively, act in the node region to prevent the Nodal signal from crossing to the right side, whereas chick Cerberus (cCer) has an unclear function in the left-side mesoderm. In this study, we investigate the transcriptional regulation and function of cCer in left-right development. By analyzing the enhancer activity of *cCer* 5′ genomic sequences in electroporated chick embryos, we identified a *cCer* left-side enhancer that contains two FoxH1 and one SMAD binding site. We show that these Nodal-responsive elements are necessary and sufficient for the activation of transcription in the left-side mesoderm. In transgenic mouse embryos, *cCer* regulatory sequences behave as in chick embryos, suggesting that the cis-regulatory sequences of *Cerberus*-related genes have diverged during vertebrate evolution. Moreover, our findings from *cCer* overexpression and knockdown experiments indicate that cCer is a negative-feedback regulator of Nodal asymmetric signaling. We propose that cCer and mouse Cerl-2 have evolved distinct regulatory mechanisms but retained a conserved function in left-right development, which is to restrict Nodal activity to the left side of the embryo.

KEY WORDS: Cerberus, FoxH1, Nodal signaling, Left-right asymmetry, Transcriptional regulation, Chick, Mouse

INTRODUCTION

Chick Cerberus (cCer; also known as Caronte) is a member of the Cerberus-Dan family of cysteine-knot-secreted proteins (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Cerberus-related proteins have been identified in other vertebrate species: the founding member *Xenopus* Cerberus (XCer) (Bouwmeester et al., 1996), zebrafish Charon (Hashimoto et al., 2004), mouse Cerberus-like (hereafter denominated Cerl-1) (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998) and mouse Cerberus-like-2 (Cerl-2; also known as Dand5 or Dante) (Marques et al., 2004). *Xenopus XCer*, mouse *Cerl-1* and chick *Cer* genes are syntenic (www.metazome.net) and, at early stages, are expressed in equivalent embryonic structures, such as the anterior endomesoderm, anterior visceral endoderm and hypoblast, respectively (Bouwmeester et al., 1996; Belo et al., 1997; Foley et al., 2000). Mouse *Cerl-1* and chick *Cer* transcripts are also detected in the anterior definitive mesendoderm (Belo et al., 1997; Rodriguez Esteban et al., 1999). However, at later stages, *Cerberus*-related genes have very distinct patterns: *XCer* expression is no longer detected, mouse *Cerl-1* transcripts are found in nascent somites and presomitic mesoderm, zebrafish *charon* and mouse *Cerl-2* are expressed around the node region (*Cerl-2* expression levels are higher on the right side), and chick *Cer* is expressed in the left paraxial and lateral plate mesoderm (Bouwmeester et al., 1996; Belo et al., 1997; Rodriguez Esteban et al., 1999; Marques et al., 2004; Hashimoto et al., 2004). The understanding of how these different patterns of expression are generated may bring some insights into the evolution of *Cerberus*-related genes and their functions in the different vertebrate species.

[1]Instituto Gulbenkian de Ciência, 2781-901 Oeiras, Portugal. [2]Institute for Biotechnology and Bioengineering, Centro de Biomedicina Molecular e Estrutural, Universidade do Algarve, 8005-139 Faro, Portugal.

*Authors for correspondence (e-mails: atavares@igc.gulbenkian.pt; jbelo@ualg.pt)

Accepted 27 March 2007

A conserved regulator of vertebrate left-right patterning is *Nodal*, a member of the transforming growth factor-β (TGF-β) family of signaling molecules that is expressed in the node region and left lateral plate mesoderm (reviewed by Hamada et al., 2002; Schier, 2003). In the mouse embryo, Nodal activity is restricted to the left side by Cerl-2 (Marques et al., 2004), by the midline barrier and by *Lefty2*, a Nodal antagonist also expressed in the left lateral plate mesoderm (reviewed by Juan and Hamada, 2001). The left-side expression of *Nodal* and *Lefty2* is directly regulated by Nodal itself. Our present findings demonstrate that *cCer* asymmetric expression is also directly activated by Nodal signaling and suggest that the cis-regulatory sequences of *Cerberus*-related genes have diverged among vertebrates.

Zebrafish *charon*, mouse *Cerl-2* and chick *Cer* have all been implicated in the determination of the left-right axis, but their functions seem to differ: zebrafish *charon* and mouse *Cerl-2* have a role in preventing Nodal signals from crossing to the right side (Hashimoto et al., 2004; Marques et al., 2004), whereas chick *Cer* was reported to have a role in transferring the positional information from the node to the left lateral plate mesoderm (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). At the molecular level, Cerberus-related proteins behave as antagonists of members of the TGF-β family (Hsu et al., 1998; Rodriguez Esteban et al., 1999; Piccolo et al., 1999; Belo et al., 2000). During left-right patterning, zebrafish Charon and mouse Cerl-2 proteins were shown to act as Nodal antagonists (Hashimoto et al., 2004; Marques et al., 2004), whereas cCer has been proposed to act as a bone morphogenetic protein (BMP) antagonist (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). Chick Cer would allow the expression of *Nodal* in the left lateral plate mesoderm by inhibiting the repressive activity of BMPs on *Nodal* transcription. However, more recent reports have shown that BMP signaling is indeed essential for the activation of Nodal expression in the left lateral plate (Piedra and Ros, 2002; Schlange et al., 2002), leaving the role of cCer in left-right patterning unexplained. Our results from overexpression and knockdown experiments demonstrate that cCer acts as a negative regulator of

Nodal expression and prevents Nodal signaling from crossing to the right side. In conclusion, we propose that chick Cer, zebrafish Charon and mouse Cerl-2 evolved different regulatory mechanisms but retained a similar role in restricting Nodal activity to the left side.

MATERIALS AND METHODS

Isolation of a *cCer* genomic clone and sequence analysis

A *cCer* genomic clone (clone MPMGc125J2191Q3 from RZPD, Germany) was isolated by screening a chicken cosmid library (RZPD no. 125) with a *cCer* sequence probe (gift from J. C. Izpisúa Belmonte, The Salk Institute, La Jolla, CA). Shorter DNA fragments of this clone were introduced into pBluescriptIIKS (Stratagene), sequenced and identified as containing the 5′, cDNA, intronic and 3′ regions of the *cCer* gene.

To recognize possible binding sites for known transcription factors, *cCer* 5′ genomic sequences were analyzed using MatInspector Professional release 7.4 (Quandt et al., 1995).

To identify the transcription initiation site(s), 5′ rapid amplification of cDNA ends was performed using total RNA from HH3-9 chick embryos and the RLM-RACE kit (Ambion). PCR products were size-fractionated by agarose gel electrophoresis, purified using a gel extraction kit (Qiagen), cloned into the pGEM-T Easy vector (Promega) and sequenced.

DNA constructs and morpholinos

cCer 5′ genomic sequences were subcloned into an enhanced green fluorescence protein (EGFP) reporter vector containing the *EGFP* coding sequence and the SV40 early mRNA polyadenylation signals from pEGFP-N3 (Clontech). Deletions or point mutations of FoxH1- and SMAD-binding elements were designed according to the literature (Zhou et al., 1998; Mostert et al., 2001) and introduced into the *Cer0.36-EGFP* construct by PCR-based site-directed mutagenesis.

For the enhancer assays, *cCer* genomic sequences were either amplified by PCR (PCR1-5) or synthesized as complementary oligonucleotides, and subcloned into the p1229-*EGFP* enhancer-less vector. This vector carries the human β-globin minimal promoter and was generated by replacing the *lacZ* gene in the β-globinlacZ BGZA or p1229 vector (Yee and Rigby, 1993) with the *EGFP* coding sequence (Clontech).

Chick expression plasmids were based on a modified pCAGGS-MCS vector (gift from D. Henrique, Instituto de Medicina Molecular, Lisbon, Portugal) (Niwa et al., 1991). The coding sequence of *Xenopus Cerberus-short* (*XCerS*) was amplified by PCR from a pCS2-*XCerS* vector (gift from S. Piccolo) (Piccolo et al., 1999). The *cCer* coding sequence (cCerCDS) was isolated by reverse transcriptase (RT)-PCR according to the published sequence (GenBank accession no. AF179484) (Rodriguez Esteban et al., 1999) and subcloned into the *Xho*I and *Not*I sites of pCAGGS-MCS.

The pCAGGS-*RFP* vector (gift from D. Henrique), carrying the cDNA of monomeric red fluorescent protein (RFP; Clontech) (Campbell et al., 2002) under the control of the CAGGS promoter, was used to control the extent and efficiency of electroporation.

To generate the luciferase (luc) reporter constructs, *cCer* regulatory sequences were amplified by PCR (using *Cer-EGFP* plasmids as template) and subcloned into the pGL2-Basic vector (Promega).

Fluorescein-tagged antisense morpholino oligonucleotides (cCer MO: 5′-CATGGTCCTGCTGATGCTGTAGATC-3′; cCer CoMO: 5′-CATcGT-CgTGCTcATGaTGTAcATC-3′, mismatches in lowercase) were designed and produced by Gene Tools. The efficacy of cCer morpholinos to inhibit the translation of *Cer-Luc* reporter constructs was tested in a cell-free transcription/translation system (see Fig. S3 in the supplementary material) (Summerton et al., 1997).

Bead implantation and whole-mount in situ hybridization

Fertilized chicken eggs (Quinta da Freiria) were incubated at 37.5°C for the appropriate period. Embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951), explanted at HH stage 4-7 (HH4-7) together with the vitelline membrane and anchored to a metacrilate ring according to the protocol of New (New, 1955). Affigel-blue beads (Bio-Rad) were soaked in Shh protein [1 mg/ml in 0.1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS); R&D Systems]; heparin acrylic beads (Sigma) were soaked in recombinant Nodal protein (0.5 mg/ml; R&D Systems); and AG1-X2 anion-exchange beads (Bio-Rad) were soaked either in SU5402 [3 mM in dimethylsulfoxide (DMSO); Calbiochem] (Mohammadi et al., 1997) or in SB-431542 (10 mM in DMSO; Tocris) (Inman et al., 2002). Treated embryos were cultured at 37.5°C in a humid chamber, fixed in 4% paraformaldehyde and processed for whole-mount in situ hybridization.

Whole-mount in situ hybridization on chicken and mouse embryos was performed as described by Liguori et al. (Liguori et al., 2003). Detailed descriptions of the RNA probes used are available from the authors on request. Embryos were developed with BM purple (Roche) for purple color and with INT/BCIP (Roche) for orange.

Embryo electroporation

Embryos were processed for New culture (New, 1955) at HH3-5 and transferred into a silicon rubber pool containing a 2 mm-square cathode (CY700-1Y electrode; Nepa Gene). The ring was then covered with warmed Hank's buffer (GibcoBRL) and the embryo was injected with a DNA solution (0.5-3 mg/ml; 0.1% Fast Green; Sigma) using a pulled glass capillary and an IM-300 microinjector (Narishige). Electroporation was performed by placing a 2 mm-square anode (CY700-2 electrode; Nepa Gene) over the embryo and applying five pulses (10 V for 50 ms at 350 ms intervals) using a square wave electroporator (ECM830; BTX). The embryo was then placed on a 30 mm Petri dish with albumen (New, 1995), incubated for the appropriate period of time (7-48h), and observed under a fluorescence stereomicroscope (Leica MZ16FA).

Luciferase reporter assay

Capped sense mouse *Nodal* mRNA was synthesized using the mMessage mMachine kit (Ambion). Eggs were obtained from *Xenopus laevis* females, cultured and microinjected as previously described (Medina et al., 2000). Embryonic stages were determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). *Xenopus* embryos were injected in each animal blastomere of the eight-cell stage with a total of 200 pg of reporter plasmid, with or without *Nodal* mRNA (50 pg), and 25 pg of pTK-Renilla luciferase. Animal caps were isolated from the blastula stage, cultured until sibling embryos reached stage 12 and lysed in 20 μL of passive lysis buffer per cap. Firefly and Renilla luciferase values were obtained by analyzing 20 μL lysate by the standard protocol provided in the Dual Luciferase Assay kit (Promega) in a luminometer (MicroLumatPlus, Berthold Technologies). Each assay was performed in triplicate and repeated independently at least twice.

Generation of transgenic mouse embryos

The transgenic mouse line *Cer2.5-EGFP* was generated by microinjection of linearized reporter construct DNA into the pronuclei of fertilized eggs from FVB mice, as described (Nagy et al., 2003). F1 embryos were collected from embryonic day (E)7.5 to E10.5, observed under a fluorescence stereomicroscope (Leica MZ16FA), fixed in 4% paraformaldehyde and processed for whole-mount in situ hybridization. For histological analyses, embryos were embedded in gelatin, cryosectioned and photographed under a fluorescence microscope (Leica DMRA2). In some slides, cell nuclei were labeled with DAPI (Molecular Probes).

RESULTS

Nodal signaling regulates *cCer* expression in the left-side mesoderm

In the chick embryo, sonic hedgehog (Shh) signaling positively regulates *Nodal* asymmetric expression (Pagán-Westphal and Tabin, 1998). In turn, Nodal was shown to induce the expression of *XCer* (Osada et al., 2000) and mouse *Cerl-1* (Waldrip et al., 1998; Brennan et al., 2001). Therefore, the induction of *cCer* expression by Shh (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999) might be mediated by Nodal. To test this hypothesis, beads soaked in recombinant mouse Nodal protein were implanted on the right side of HH6 chick embryos and *cCer* expression was examined by whole-mount in situ hybridization. Indeed, ectopic expression of *cCer* was observed in the right-side mesoderm of these embryos

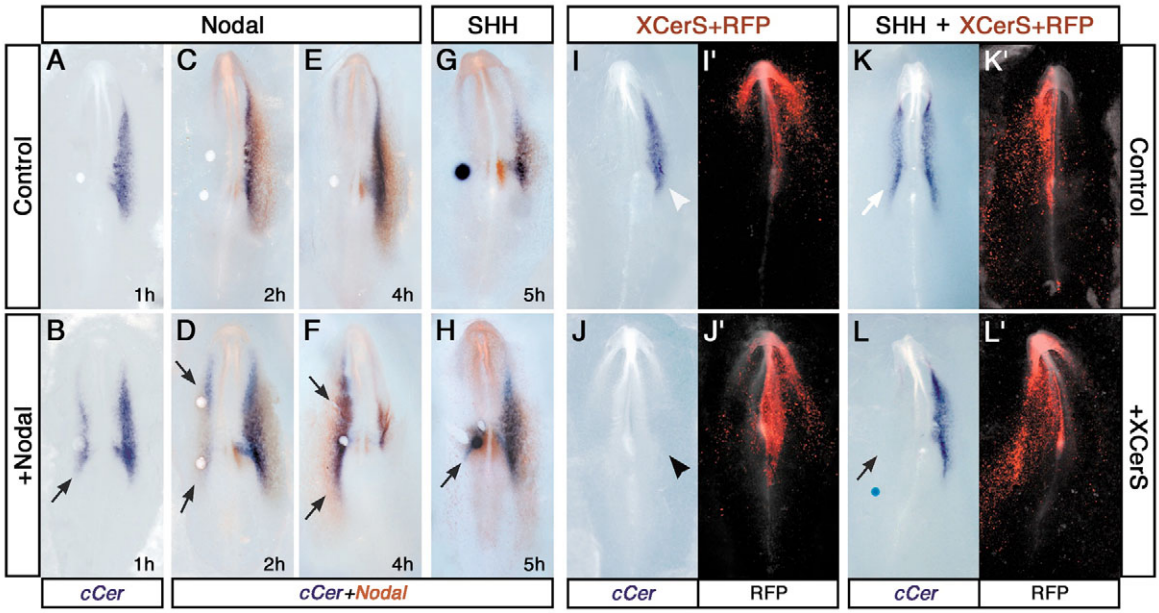

Fig. 1. Regulation of chick *Cer* and *Nodal* expression by Nodal and Shh signaling pathways. (**A-H**) Beads soaked in phosphate-buffered saline (PBS, control; A,C,E,G), Nodal protein (B,D,F) or Shh protein (H) were implanted on the right side at Hamburger and Hamilton stage 7 (HH7; A-F) or HH4 (G,H). Chick embryos were fixed after 1 hour (A,B), 2 hours (C,D), 4 hours (E,F) or 5 hours (G,H) and processed for single-label [chick *Cer* (*cCer*); A,B] or double-label (*cCer* and *Nodal*; C-H) whole-mount in situ hybridization (*cCer*, purple; *Nodal*, orange). Nodal protein induced the right-side expression of *cCer* transcripts in less than 1 hour (B; arrow), and *Nodal* transcripts in approximately 2 hours (D; arrows). After 4 hours, both *cCer* and *Nodal* expression levels were higher on the right than on the left side (F; arrows). On the other hand, Shh protein took approximately 5 hours to induce the transcription of both *cCer* and *Nodal* (H; arrow). (**I-L**) *cCer* transcripts detected by whole-mount in situ hybridization. (**I′**,**J′**,**K′**,**L′**) Merge of bright-field and RFP red fluorescence images. (I-J′) Effect of the Nodal antagonist *Xenopus* CerS (XCerS) on *cCer* expression. (J,J′) Chick embryos were electroporated with pCAGGS-*XCerS* on the left side of the node at HH4 (i.e. in the cells that express *Nodal*). pCAGGS-*RFP* was electroporated alone (control; I,I′) or with pCAGGS-XCerS (J,J′), and used to label the populations of electroporated cells. In contrast to the control electroporation (I; arrowhead), the inhibition of Nodal by XCerS suppressed the left-sided expression of *cCer* (J; arrowhead). (K-L′) Effect of the Nodal antagonist XCerS on Shh-induced *cCer* expression. HH4 chick embryos were electroporated with pCAGGS-*RFP* alone (control; K,K′) or co-electroporated with pCAGGS-*RFP* and pCAGGS-*XCerS* (L,L′), and grafted on the right side with beads soaked in Shh protein. Ectopic induction of *cCer* expression by Shh (K; arrow) was suppressed by the Nodal inhibitor XCerS (L; arrow). All embryos are viewed from the ventral side. *cCer*, chick *Cer*; RFP, red fluorescent protein; *XCerS*, *Xenopus CerS*.

(81%, *n*=33, Fig. 1A-F). *cCer* was also induced in embryos electroporated with a *Dorsalin-Nodal* expression construct (pCAGGS-*DcNodal*) (Bertocchini and Stern, 2002), but with less efficiency (35%, *n*=23, data not shown).

It has been suggested that Shh induces *Nodal* in the chick lateral plate mesoderm via a secondary signal (Pagán-Westphal and Tabin, 1998). In the mouse embryo, *Nodal* expression in the left lateral plate mesoderm is directly activated by Nodal protein produced in the node (Saijoh et al., 2003; Yamamoto et al., 2003). Accordingly, we observed that exogenous Nodal protein is able to activate *Nodal* expression in the right lateral plate mesoderm of chick embryos (68%, *n*=19, Fig. 1C-F). Nodal protein induced ectopic *cCer* expression in less than 1 hour (73%, *n*=11, Fig. 1B and data not shown) and *Nodal* expression in approximately 2 hours (71%, *n*=7, Fig. 1D), whereas beads soaked in Shh protein started to activate *cCer* and *Nodal* transcription no sooner than 4-5 hours after implantation (66%, *n*=6, Fig. 1H; 0% 2 hours after implantation, *n*=5, data not shown). Taken together, these observations suggest that the transcription of *cCer* and *Nodal* is directly regulated by Nodal.

To determine whether endogenous Nodal signaling is necessary for *cCer* expression, chick embryos were electroporated with a pCAGGS expression vector containing the Nodal-specific antagonist *Xenopus Cerberus-short* (*XCerS*) (Piccolo et al., 1999; Bertocchini and Stern, 2002). Embryos were co-electroporated with pCAGGS-red fluorescent protein (RFP) and initially scored for the co-localization of RFP fluorescence and *XCerS* mRNA (data not shown). As expected, the inhibition of Nodal by XCerS resulted in the downregulation of *cCer* (89%, *n*=9, Fig. 1J) and *Nodal* (85%, *n*=13, data not shown), whereas control electroporations had no effect (*n*=10, Fig. 1I). Similarly, *cCer* expression was also repressed by SB-431542, an inhibitor of Nodal receptors (88%, *n*=16, see Fig. S1 in the supplementary material). Therefore, we conclude that endogenous Nodal signaling is required for normal activation of *cCer* and *Nodal* expression in the left lateral plate mesoderm. In addition, ectopic induction of *cCer* by Shh protein was inhibited by XCerS (86%, *n*=7, Fig. 1L), which demonstrates that Nodal signaling is required for the activation of *cCer* expression by Shh.

Identification of the *cCer* left-side enhancer

To investigate further whether *cCer* is a direct target of Nodal signaling, we analyzed the regulatory sequences responsible for *cCer* transcription in the left-side mesoderm. For this, *cCer* 5′ genomic sequences of different lengths were subcloned into an enhanced green fluorescence protein (EGFP) reporter vector (*Cer-EGFP* constructs) and introduced into chick embryos by

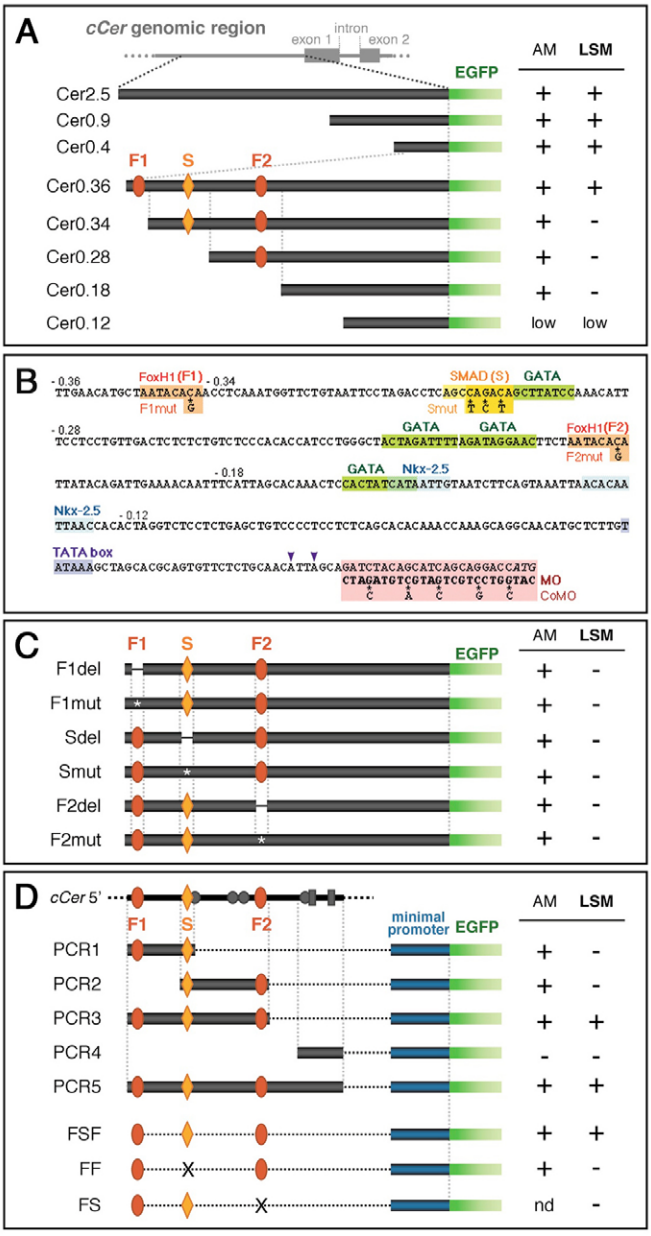

Fig. 2. Identification of the chick *Cer* left-side enhancer. (**A**) Deletion analysis of chick *Cer* (*cCer*) cis-regulatory sequences. The genomic organization of *cCer* is depicted at the top. *cCer* 5′ sequences (black boxes) were fused to the reporter *EGFP* gene (green boxes) to determine the activity of each DNA fragment. The FoxH1 elements (red; F1 and F2) and the SMAD element (orange; S) are depicted in the reporter constructs. The presence (+) or absence (–) of *EGFP* expression in the anterior mesendoderm and its derivatives (AM) and in the left-side mesoderm (LSM) from electroporated chick embryos is listed on the right. Each result is representative of at least 12 embryos. LSM expression was disrupted in embryos electroporated with *Cer0.34* or shorter constructs. *Cer0.12-EGFP* expression was very weak and ubiquitous (low). (**B**) Nucleotide sequence of the 5′-flanking region of *cCer*. Binding sites for the transcription factors FoxH1 (F1 and F2; orange), SMAD (S; yellow), GATA (green) and Nkx-2.5 (light blue), and a putative TATA box (purple), are outlined. Two transcription initiation sites were identified by RLM-RACE at positions –26 and –29 upstream of the ATG (arrowheads). Point mutations were introduced into the F1, S and F2 sites, as indicated. The morpholino antisense oligo sequence (MO) and its control oligo with five mismatches (CoMO) are outlined in pink. (**C**) Site-directed mutagenesis analysis of FoxH1- and SMAD-binding elements. LSM expression was specifically abolished in embryos transfected with constructs carrying deletions or mutations (*) in the FoxH1 (F1del, F1mut, F2del and F2mut) or SMAD (Sdel and Smut) elements. (**D**) Enhancer analysis of potential regulatory sequences of *cCer*. Fragments of the *cCer* 5′ region (PCR1-5) and sequences of the FoxH1 and SMAD elements (FSF, FF and FS) were subcloned into an enhancer-less vector carrying the human beta-globin minimal promoter (blue boxes) upstream of the *EGFP* coding sequence. LSM expression was detected in embryos electroporated with the PCR3, PCR5 and FSF constructs (which contained all of the F1, F2 and S elements), but not in those electroporated with the PCR1, PCR2, PCR4, FF and FS constructs (which lacked at least one of those sites). EGFP fluorescence was observed in the AM of embryos electroporated with each of the EGFP reporter constructs tested, with the exception of PCR4. FS-EGFP expression was not tested in the AM cells (nd). +/–, presence/absence of *EGFP* expression; AM, anterior mesendoderm and its derivatives; CoMo, control morpholino oligo sequence; EGFP, enhanced green fluorescence protein; F1/F2, FoxH1-binding sites; LSM, left-side mesoderm; MO, morpholino antisense oligo sequence; nd, not tested. S, SMAD-binding site.

microinjection and electroporation in New culture (New, 1955). A representation of these constructs and their electroporation results are summarized in Fig. 2A.

Our initial results showed that a 2.5 kb DNA fragment upstream of the ATG of *cCer* (Cer2.5) was able to drive the expression of *EGFP* into the cell populations that express *cCer* (i.e. the anterior mesendoderm and left paraxial and lateral plate mesoderm) (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Fluorescent cells are later detected in the foregut and heart (data not shown), which are derivatives of the chick anterior mesendoderm. The subsequent analysis of *EGFP* expression driven by shorter fragments (Cer0.9, Cer0.4 and Cer0.36) revealed a similar pattern (Fig. 3A,C, and data not shown). However, left-side expression was specifically disrupted in embryos electroporated with *Cer0.34-EGFP* and shorter constructs (data not shown). These observations indicate that the *cCer* left-side enhancer is located in the 360 bp 5′ region and that the –360 to –340 sequence contains an essential regulatory element.

FoxH1 and SMAD elements are essential for *cCer* enhancer activity in the left-side mesoderm

To confirm that *cCer* left-side expression is directly activated by Nodal, we first analyzed the *cCer* left-side enhancer sequence and looked for the presence of FoxH1- and SMAD-binding sites. These transcription factors are nuclear effectors of the Nodal signaling pathway (reviewed in Schier and Shen, 2000) and were shown to directly regulate the asymmetric expression of the *Nodal*, *Lefty2* and *Pitx2* genes (Saijoh et al., 2000; Osada et al., 2000; Yashiro et al., 2000; Shiratori et al., 2001). Sequence analysis of the *cCer* 360 bp 5′ region (Cer0.36) using MatInspector software (Professional

release 7.4) revealed the presence of a possible TATA box at –60, and consensus binding sites for several putative regulators of *cCer* transcription, including two FoxH1 and one SMAD element (Fig. 2B).

To determine whether the FoxH1 elements (F1 and F2) or the SMAD element (S) are necessary for the regulation of *cCer* asymmetric expression, we constructed *Cer0.36-EGFP* reporter vectors containing deletions (del) or mutations (mut) in each one of those sites that were previously shown to disrupt their activity (F1del, F1mut, F2del, F2mut, Sdel and Smut) (Zhou et al., 1998; Mostert et al., 2001) (indicated in Fig. 2B). Each of the F1del, F1mut, F2del, F2mut, Sdel and Smut constructs was electroporated into chick embryos, and EGFP fluorescence was analyzed both in the anterior mesendoderm and in the left-side mesoderm (results are summarized in Fig. 2C). All constructs were able to drive *EGFP* expression in the anterior mesendoderm (Fig. 3B and data not shown), but left-side expression was specifically abolished (Fig. 3D and data not shown). These observations demonstrate that the FoxH1 and SMAD sites in *Cer0.36* are essential for the induction or maintenance of left-side transcription.

In addition, the functions of the FoxH1 and SMAD elements in the *cCer* left-side enhancer were quantified in luciferase reporter assays with *Xenopus* animal caps. The *Cer0.36* reporter construct was clearly activated in the presence of Nodal (Fig. 3I). However, luciferase activity was reduced with the introduction of mutations in one of the FoxH1 or SMAD elements (F1mut, Smut and F2mut constructs; Fig. 3I). Taken together, our results indicate that the *cCer* left-side enhancer is directly activated by the Nodal-FoxH1/SMAD signaling pathway.

FoxH1 and SMAD elements are sufficient to activate the *cCer* left-side enhancer

We next investigated whether the FoxH1 and SMAD elements in the *cCer* left-side enhancer are sufficient to induce left-side expression. For this, potential regulatory sequences were subcloned into enhancer-less vectors that contain the human beta-globin minimal promoter upstream of either the *EGFP* or the luciferase reporter gene. The potential enhancer sequences tested were either shorter fragments of *Cer0.36* (PCR1-5) or combinations of individual FoxH1 (F) and SMAD (S) elements (FSF, FF and FS; results are summarized in Fig. 2D). Embryos electroporated with PCR1, PCR2, PCR4, FF or FS did not display *EGFP* expression in the left-side mesoderm (Fig. 3F,H, and data not shown). By contrast, asymmetric expression was detected in embryos electroporated with the PCR3, PCR5 or FSF constructs (Fig. 3E,G, and data not shown). Accordingly, luciferase activities of the reporter constructs that lack one of the FoxH1 or SMAD elements (PCR1, PCR2, FS and FF) were severely reduced when compared with those of PCR3 or FSF (Fig. 3J). These observations indicate that the FSF module in the *cCer* left-side enhancer is sufficient to activate asymmetric expression.

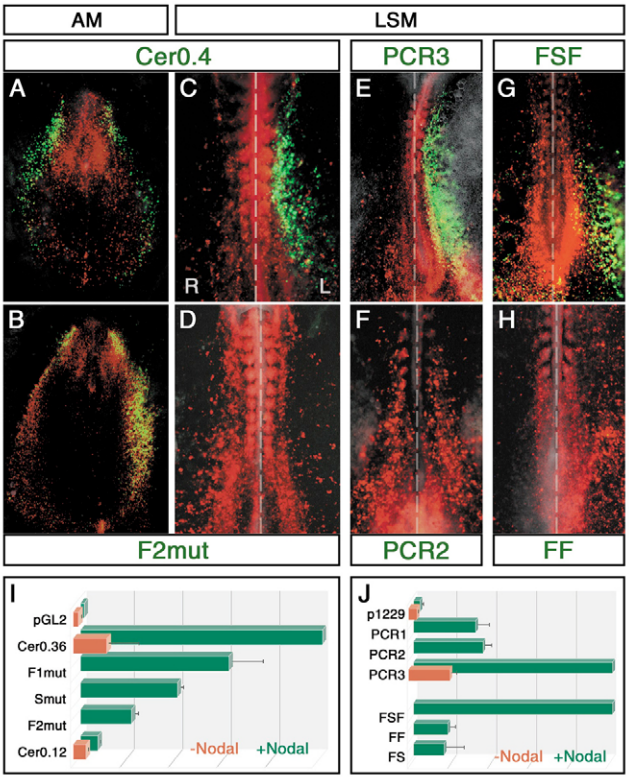

Fig. 3. Expression analysis of *Cer-EGFP* reporter constructs. (**A-H**) *Cer-EGFP* reporter expression in electroporated chick embryos. Different embryos were co-transfected with one *Cer-EGFP* reporter construct (green fluorescence) and the pCAGGS-*RFP* construct (positive control; red fluorescence). (A,C) Cer0.4-EGFP; (B,D) F2mut; (E) PCR3; (F) PCR2; (G) FSF; (H) FF (see Fig. 2 for construct details). (A,B) EGFP fluorescence was observed in the anterior mesendoderm (AM) of embryos electroporated with Cer0.4 (A) and F2mut (B) reporter constructs. Embryos were electroporated at Hamburger and Hamilton stage 3 (HH3) and fixed at HH6. (C-H) Asymmetric *EGFP* expression was detected in the left-side mesoderm (LSM) of embryos electroporated with Cer0.4 (C), PCR3 (E) and FSF (G), but not in those electroporated with the F2mut (D), PCR2 (F) or FF (H) reporter constructs. Embryos were electroporated at HH4-5 and fixed at HH8-9. Dashed line separates the right and left sides of the embryos. (**I**,**J**) *Cer-Luc* reporter activity in *Xenopus* animal cap luciferase assays. Luciferase reporter plasmids containing the indicated wild-type or mutant fragments of chick *Cer* (*cCer*) regulatory sequences were injected into *Xenopus* embryos in the absence (orange) or presence (green) of *Nodal* mRNA. Data are relative to the highest luciferase activity values (Cer0.36+Nodal in I; PCR3+Nodal and FSF+Nodal in J). The activities of reporter constructs that either lack one of the FoxH1 elements (F1mut, F2mut, PCR1, PCR2 or FS) or lack the SMAD element (Smut and FF) were reduced. AM, anterior mesendoderm; L, left; LSM, left-side mesoderm; R, right.

Regulation of the *cCer* left-side enhancer by Nodal signaling

Asymmetric expression of *cCer* is induced by Shh on the left side and repressed by fibroblast growth factor 8 (FGF8) on the right side (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). To investigate whether the *cCer* enhancer region is also regulated by these signaling molecules, chick embryos were electroporated with *Cer0.4-EGFP* and grafted on the right side of the node with beads soaked either in Shh protein or in the FGF receptor-1 inhibitor SU5402. In addition to the expected left-side pattern, *EGFP* expression was activated on the right side both by the Shh protein (100%, *n*=10, Fig. 4C) and by SU5402 (62%, *n*=13, Fig. 4D). These observations demonstrate that the *cCer* left-side enhancer is regulated by Shh and FGF signaling in the same way as *cCer* expression.

To confirm that *cCer* enhancer activity is regulated by Nodal, embryos were electroporated with the *Cer0.4-EGFP* reporter construct and grafted with beads soaked in Nodal protein. As expected, Nodal was able to ectopically induce *EGFP* expression in the right-side mesoderm (100%, *n*=11, Fig. 4B; compare with

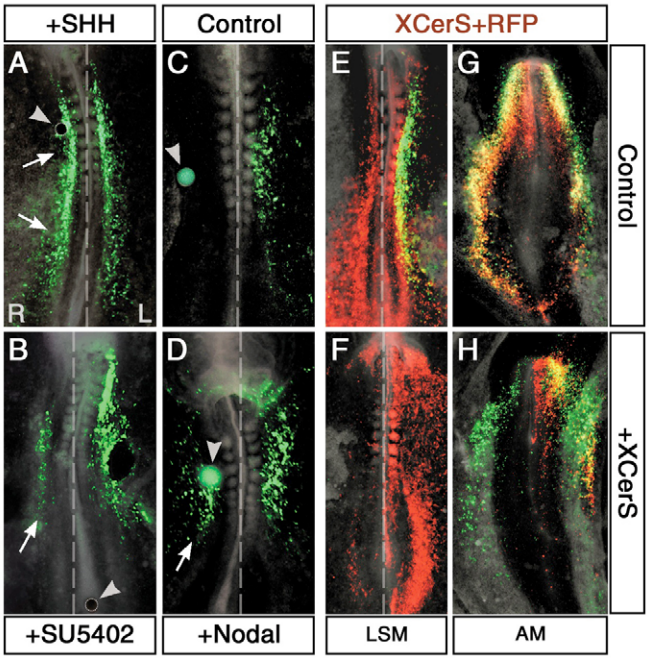

Fig. 4. Regulation of the chick *Cer* left-side enhancer by Shh, FGF and Nodal signaling pathways. (**A-F**) Analysis of *Cer-EGFP* expression in the left-side mesoderm (LSM) of embryos electroporated at HH4-5 and fixed at HH8-9. (**G**,**H**) Analysis of *Cer-EGFP* expression in the anterior mesendoderm (AM) of embryos electroporated at HH3 and fixed at HH6. (A-D) Chick embryos were electroporated with *Cer0.4-EGFP* and grafted with beads (arrowheads) soaked in Shh protein (A), the FgfR1 inhibitor SU5402 (B), phosphate-buffered saline (PBS, control; C) or Nodal protein (D). *EGFP* expression was ectopically induced on the right side by Shh, SU5402 and Nodal (arrows). (E-H) Effect of the Nodal antagonist *Xenopus* CerS (XCerS) on chick *Cer* (*cCer*) left-side enhancer activity. Chick embryos were electroporated either with pCAGGS-*RFP* and *PCR5-EGFP* (control; E,G) or with these plus pCAGGS-*XCerS* (F,H). XCerS repressed the transcription of *PCR5-EGFP* in the LSM (E,F), whereas it had no effect on AM expression (G,H). AM, anterior mesendoderm; LSM, left-side mesoderm; XCerS, *Xenopus* CerS.

control, *n*=5, Fig. 4A). Conversely, when embryos were co-electroporated with the *PCR5-EGFP* reporter construct and the Nodal antagonist *XCerS* (pCAGGS-*XCerS*), *cCer* enhancer activity was specifically repressed in the left-side mesoderm (64%, *n*=11, Fig. 4F; compare with control, *n*=4, Fig. 4E). XCerS did not have an effect on anterior mesendoderm expression (*n*=4, Fig. 4G,H). These observations indicate that Nodal signaling is required for the regulation of *cCer* transcription in the left-side mesoderm.

cCer regulatory region is active in the left-side mesoderm of transgenic mouse embryos

Chick and mouse *Cerberus*-related genes have both coincident and distinct domains of expression during embryonic development. At early stages, chick *Cer* and mouse *Cerl-1* are both expressed in equivalent embryonic structures, such as the anterior mesendoderm (Rodriguez Esteban et al., 1999; Belo et al., 1997). However, at later stages, chick *Cer* is expressed in the left-side mesoderm (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999) (see also Fig. 1A), whereas mouse *Cerl-1* expression is found in the rostral domain of the nascent somites and presomitic mesoderm (Belo et al., 1997) and mouse *Cerl-2* is expressed in the node region (Marques et al., 2004). In order to determine whether the upstream regulators of *cCer* expression are conserved in mouse, we generated a transgenic line carrying the *cCer* regulatory region (*Cer2.5-EGFP*) and analyzed reporter gene expression in mouse embryos at different stages. At E7.5, EGFP fluorescence was detected in the anterior mesendoderm (data not shown), an expression domain common to chick *Cer* and mouse *Cerl-1* genes. However, at E8.5, *EGFP* was expressed in the left lateral plate mesoderm (Fig. 5A,B), which is a *cCer*-specific pattern. As in *Cer-EGFP*-electroporated chick embryos, fluorescent cells were also found in the foregut and heart of E8.5 transgenic embryos (Fig. 5Aa,B). These results indicate that the upstream regulators of *cCer* expression are present not only in tissues that express both *cCer* and mouse *Cerl-1* (i.e. anterior mesendoderm), but also in the mouse left-side mesoderm, a region that expresses *cCer* but not the mouse Cerl genes.

In the mouse embryo, the asymmetric expression of both *Nodal* and *Lefty2* is directly regulated by Nodal signaling (Saijoh et al., 2000; Saijoh et al., 2003; Yamamoto et al., 2003). In *Cer2.5-EGFP* mouse embryos, *EGFP* mRNA expression is exclusively detected in the left lateral plate mesoderm at E8.25, and coincides with the expression patterns of *Nodal* (Fig. 5C,C′) and *Lefty2* (Fig. 5D,D′), which reinforces the hypothesis that *cCer* regulatory sequences are directly regulated by Nodal.

Nodal signaling is negatively regulated by cCer

In the chick embryo, *Lefty* is expressed in the midline (as is mouse *Lefty1*) and in a small posterior domain of the left lateral plate mesoderm at late stages, whereas the *cCer* expression pattern is much more similar to that of mouse *Lefty2* in the left-side mesoderm (Rodriguez Esteban et al., 1999; Ishimaru et al., 2000). Like Lefty proteins, Cerberus-related molecules were shown to act as Nodal antagonists in zebrafish (Hashimoto et al., 2004), *Xenopus* (Hsu et al., 1998; Piccolo et al., 1999), chick (Bertocchini and Stern, 2002) and mouse (Belo et al., 2000; Marques et al., 2004) embryos. Therefore, we proposed that cCer has taken the role of mouse Lefty2 in the left-side mesoderm, and acts to restrict the range of Nodal signaling. To test this hypothesis, we have performed *cCer* overexpression and knockdown experiments in chick embryos. Because *Nodal* transcription is autoregulated, *Nodal* expression was analyzed as a readout of Nodal signaling.

Chick embryos electroporated on the left side with a pCAGGS expression vector containing the *cCer* coding sequence (pCAGGS-*cCerCDS*) showed a dramatic reduction or absence of *Nodal* expression in the left lateral plate mesoderm, but not in the node region (95%, *n*=20, Fig. 6B; compare with control, *n*=4, Fig. 6A). On the other hand, when *cCer* was misexpressed on the right side, *Nodal* was never ectopically induced (*n*=18, Fig. 6C) and was downregulated only in one embryo (6%, *n*=18, data not shown). In this embryo, it is possible that the cCer protein had traveled from the right to the left side, where it inhibited the Nodal signal. In addition, the expression of the Nodal target gene *Pitx2* was downregulated by cCer on the left side (56%, *n*=16, data not shown), whereas it was never induced on the right side (*n*=11, data not shown). At older stages, chick embryos showed reversed heart looping when *cCer* was overexpressed on the left side (47%, *n*=15, data not shown), but not when *cCer* was misexpressed on the right side (*n*=7) nor in control electroporations (*n*=4). Taken together, these observations suggest that cCer may act as a negative regulator of Nodal signaling.

To investigate the effect of cCer downregulation on *Nodal* expression, fluorescein-tagged morpholino oligonucleotides against *cCer* (MO) or against a related sequence (five mismatches; CoMO; see Fig. 2B) were electroporated into the future left-side mesoderm

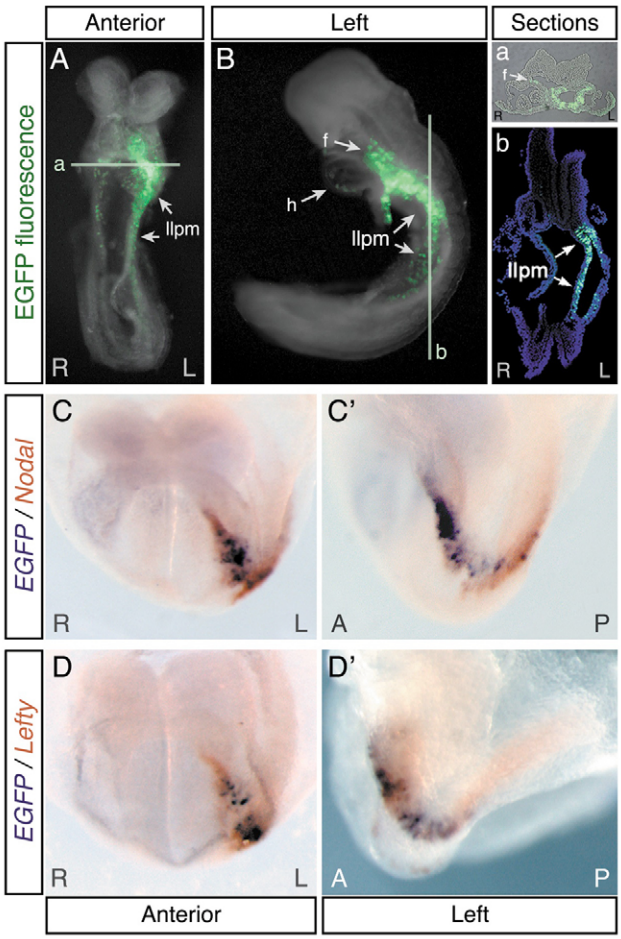

Fig. 5. Chick *Cer* regulatory regions are able to drive *EGFP* expression in the left lateral plate mesoderm of mouse embryos. (**A**,**B**) E8.5 *Cer2.5-EGFP* transgenic mouse embryos in ventral (A) and left-side (B) views. (**Aa**) Transverse section of embryo in A (line). (**Bb**) Longitudinal section of embryo in B (line). Cell nuclei are labeled with DAPI (blue). Green fluorescence was asymmetrically detected in the left lateral plate mesoderm (llpm; A,B), both in the splanchnopleure and in the somatopleure (Aa,Bb). Fluorescent cells were also found in the foregut (f) and heart (h; B,Aa). (**C-D'**) Expression patterns of *EGFP* (purple) and *Nodal* (orange; C,C′) or *Lefty1,2* (orange; D,D′) in E8.25 transgenic mouse embryos detected by double whole-mount in situ hybridization. (C,D) Anterior views. (C′,D′) Left-side views. The expression domain of *EGFP* overlapped with *Nodal* and *Lefty2* in the left lateral plate mesoderm. A, anterior; f, foregut; h, heart; L, left; llpm, left lateral plate mesoderm; P, posterior; R, right.

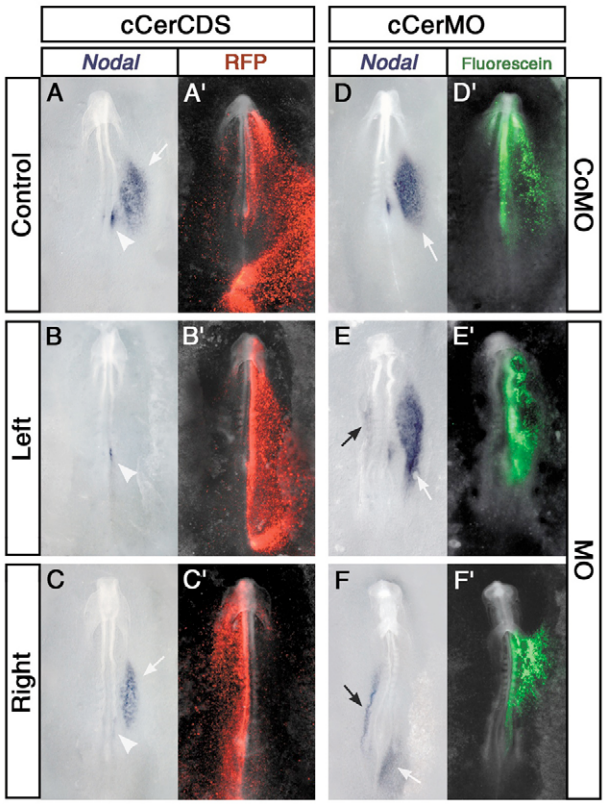

Fig. 6. Regulation of *Nodal* expression by chick Cer. (**A-C'**) Effect of chick *Cer* (*cCer*) overexpression. Chick embryos were electroporated with pCAGGS-*cCerCDS* (coding sequence) on the left (A-B′) or right (C,C′) side at Hamburger and Hamilton stage 4 (HH4) and fixed at HH8. pCAGGS-*RFP* was electroporated alone (control; A,A′) or with pCAGGS-*cCerCDS* (B-C′). (A-C) *Nodal* transcripts detected by whole-mount in situ hybridization. (A′,B′,C′) Merge of bright-field and RFP fluorescence images. *cCer* overexpression on the left side suppressed *Nodal* expression (A) in the left lateral plate mesoderm (B; arrow in A), whereas *cCer* misexpression on the right side had no effect (C; arrow). *Nodal* transcripts were always detected in the node (A-C; arrowheads). (**D-F'**) Effect of *cCer* knockdown. HH4-6 chick embryos were electroporated on the left side with fluorescein-tagged morpholinos (MO) and fixed at HH8-11. (D-F) *Nodal* transcripts detected by whole-mount in situ hybridization. (D′,E′,F′) Merge of bright-field and fluorescein green fluorescence images. (E,F) *Nodal* expression was ectopically induced by *cCer* MO in the right lateral plate mesoderm (black arrows), whereas it was normal in the left side (white arrows). Electroporation of a control morpholino (CoMo) did not perturb *Nodal* left-side expression (D; white arrow). *cCer*, chick *Cer*; *cCerCDS*, chick *Cer* coding sequence.

of HH4-6 chick embryos. At HH8-11, *Nodal* transcription was ectopically induced on the right side of cCer morphant embryos (MO: 51%, *n*=37, Fig. 6E,F versus CoMO: 7%, *n*=27, Fig. 6D). In nine HH10-11 morphant embryos, *Nodal* expression on the right was higher than on the left side (Fig. 6F). This observation can be explained by a right-side-biased amplification of Nodal signaling, as predicted by the self-enhancement and lateral inhibition (SELI) model in the absence of Nodal inhibitors (Nakaguchi et al., 2006). The *Lefty* midline expression domain was normal in cCer MO-treated embryos (*n*=26, see Fig. S2 in the supplementary material), suggesting that the midline barrier was not affected. At older stages, *cCer* knockdown resulted in the inversion of heart looping (43%, *n*=7 versus CoMO: 0%, *n*=5, data not shown). These results indicate that the main function of cCer in the left-side mesoderm is to prevent Nodal signaling from crossing to the right side.

DISCUSSION

cCer asymmetric expression is regulated by Nodal

We have demonstrated that Nodal is sufficient and necessary for the induction of *cCer* expression (Fig. 1). However, previous studies have reported that Nodal is unable to activate *cCer* expression in the right lateral plate mesoderm (Rodriguez Esteban et al., 1999;

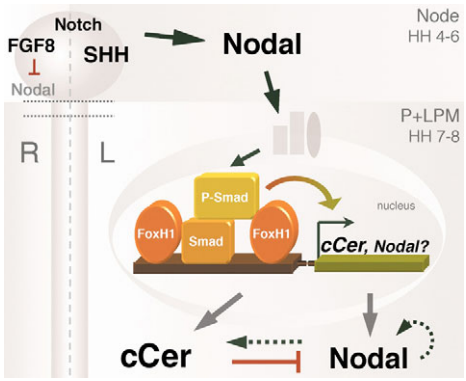

Fig. 7. Proposed model of the regulation and function of chick Cer in the left-side mesoderm. At Hamburger and Hamilton stage 5-6 (HH5-6), the early expression of *Nodal* in the node is activated on the left side by Notch and Shh signaling pathways, and is repressed on the right side by Fgf8. At HH7, the Nodal protein released by the node directly activates chick *Cer* (*cCer*) and *Nodal* expression in the adjacent left paraxial and lateral plate mesoderm (P+LPM). Nodal signal is transduced into the phosphorylation of SMAD2 and/or SMAD3, which then bind SMAD4, translocate into the nucleus and synergize with the FoxH1 transcription factor in the activation of *cCer* transcription. At HH8, Nodal protein produced by the P+LPM cells upregulates *cCer* and *Nodal* expression throughout the left lateral plate mesoderm (broken arrows). cCer protein is then required to downregulate the Nodal signal in the left lateral plate mesoderm and prevent it from crossing to the right side of the chick embryo. L, left; R, right.

Yokouchi et al., 1999; Zhu et al., 1999). The inconsistency between these and our results may be a consequence of the usage of different Nodal overexpression methods: we grafted beads soaked in active Nodal protein (mature form; R&D Systems), whereas others used retroviral vectors carrying a Bmp4-Nodal fusion protein that were introduced either by direct injection (Rodriguez Esteban et al., 1999) or by implantation of expressing cell pellets (Yokouchi et al., 1999; Zhu et al., 1999). In fact, induction of ectopic *cCer* expression was much less efficient when we used a Dorsalin-Nodal fusion construct (34%, versus 81% with Nodal protein beads) and was never detected when we overexpressed chick Nodal complete cDNA (n=10, data not shown). Therefore, it is possible that the proprotein convertase required for Nodal maturation is present at low levels in the right side of the chick embryo (Constam and Robertson, 2000), or that the Nodal protein encoded by these constructs is less stable than the recombinant protein (Le Good et al., 2005).

In zebrafish, *Xenopus* and mouse embryos, *Nodal* expression in the left lateral plate mesoderm is directly induced by Nodal protein released by the node (Long et al., 2003; Osada et al., 2000; Saijoh et al., 2003; Yamamoto et al., 2003). Accordingly, our results indicate that Nodal signaling positively regulates *Nodal* expression in the left lateral plate in the chick embryo (Fig. 1). This observation supports the hypothesis that Nodal itself is the intermediary signal that transfers the asymmetric information from the node to the lateral plate (Pagán-Westphal and Tabin, 1998).

cCer left-side enhancer is regulated by Nodal signaling via FoxH1 and SMAD elements

Here we have provided evidence that the FoxH1 and SMAD elements present in the *cCer* left-side enhancer are essential and sufficient for the activation of asymmetric expression (Figs 2, 3). FoxH1 and SMAD transcription factors are nuclear mediators of Nodal signaling (reviewed by Schier and Shen, 2000). As expected, Nodal is necessary and sufficient to activate the *cCer* left-side enhancer (Fig. 4). These observations, together with the evidence that *cCer* asymmetric expression is activated by Nodal (Fig. 1), indicate that Nodal signaling directly regulates *cCer* transcription in the left-side mesoderm via the activity of FoxH1 and SMAD factors. In the future, the isolation of chick FoxH1 and the investigation of its direct binding and activation of the FoxH1 elements present in the *cCer* left-side enhancer may bring additional support to these results.

cCer restricts the range of Nodal signaling to the left side

Previous reports have proposed that cCer is able to induce ectopic *Nodal* expression on the right side by antagonizing the repressive activity of BMPs on *Nodal* transcription (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). However, BMPs can have opposite effects on *Nodal* expression: Bmp4 is a negative regulator of Nodal in the right side of the chick node at early stages (HH5-6) (Monsoro-Burq and Le Douarin, 2001), whereas Bmp2 positively regulates Nodal signaling in the lateral plate mesoderm at later stages (HH7-8) (Piedra and Ros, 2002; Schlange et al., 2002). Because cCer was introduced at early stages (HH6) on the right side of the node (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999), it might be inducing *Nodal* expression by blocking the inhibitory function of Bmp4 in the node region. In fact, when *cCer*-expressing cells were implanted at later stages (HH7-8) in the lateral plate, *Nodal* expression was not affected (Zhu et al., 1999).

In our hands, *cCer* misexpression in the node region (n=7, data not shown) or right lateral plate (Fig. 6C) was never able to induce *Nodal*, whereas *cCer* overexpression on the left side actually repressed *Nodal* (Fig. 6B). Conversely, Nodal was ectopically expressed on the right side of *cCer*-knockdown embryos (Fig. 6E,F). These findings revealed that *cCer* acts as a negative regulator of Nodal signaling. Similar results have been obtained with the Nodal antagonist Lefty: Nodal activity was repressed by the ectopic expression of chick Lefty (Rodriguez Esteban et al., 1999) or mouse Lefty1 or Lefty2 (Yoshioka et al., 1998) in the chick embryo, whereas it was upregulated on the right side of Lefty2 mutant mice (Meno et al., 2001). Given the similarities between the expression patterns and functions of chick *Cer* and mouse *Lefty2* (reviewed by Juan and Hamada, 2001), we propose that cCer has taken the role of mouse Lefty2 in left-right patterning, and acts in addition to the midline barrier to confine Nodal signaling to the left side.

Feedback model of cCer and Nodal regulation

Taken together, our findings suggest a feedback mechanism by which Nodal signaling is restricted in the left lateral plate mesoderm of the chick embryo (Fig. 7). During the establishment of the left-right axis, *Nodal* expression is first activated in the left perinodal region by the Notch and Shh signaling pathways (reviewed by Raya and Izpisua-Belmonte, 2004). This initial Nodal signal directly induces *cCer* expression via the activation of the *cCer* left-side enhancer by FoxH1 and SMAD transcription factors. We hypothesize that SMAD2 and/or SMAD3 regulate *cCer* transcription, because they are thought to transduce Nodal signal in the left-side mesoderm (reviewed by Schier, 2003) and phospho-SMAD2 has been detected in the chick lateral plate mesoderm (Faure et al., 2002). Additionally, we propose that, as in the mouse embryo (reviewed in Hamada et al., 2002), Nodal also activates its own transcription, leading to the amplification of *Nodal* and *cCer*

expression throughout the left lateral plate. The partial overlap of the *Nodal* and *cCer* expression domains is possibly determined by functional differences in their regulatory regions and/or in Nodal and cCer diffusion rates, as proposed for the mouse Nodal and Lefty2 proteins (Nakaguchi et al., 2006). Together with the midline barrier, cCer has a key role in preventing the Nodal signal from crossing to the right side. Ultimately, the negative-feedback regulation of Nodal signaling by cCer results in the downregulation of *Nodal* and *cCer* expression in the left lateral plate mesoderm. Further support for this model may come from the analysis of chick *Nodal* transcriptional regulation as well as from the investigation of the diffusion rates and stability of Nodal and cCer proteins.

Evolution of *Cerberus*-related genes: divergence of gene regulation but conservation of function in left-right patterning

Unlike other known *Cerberus*-related genes, *cCer* is expressed on the left side of the paraxial and lateral plate mesoderm. Variations in the expression patterns of orthologous genes may arise either from the presence of particular cis-regulatory elements in their genomic sequence, or from the existence of differences in the localization and activation status of their upstream regulators, or both. Cross-species studies of cis-regulatory sequences are likely to help distinguish between these two hypotheses. In our study, the analysis of *Cer-EGFP* transgenic mouse embryos revealed that the upstream regulators of the *cCer* left-side enhancer (i.e. Nodal) are present in the mouse left lateral plate mesoderm, and suggested that the regulatory regions of *Cerberus*-related genes have diverged in chick and mouse. In agreement with this, the comparison between the *cCer* left-side enhancer and non-coding sequences of human, mouse, *Xenopus* and *Fugu Cerberus*-related genes using ConSite and VISTA programs was unable to detect any conserved FoxH1-binding sites or other common regulatory elements. FoxH1- and SMAD-binding sites are indeed present in the asymmetric enhancers of several left-side-specific genes, such as the ascidian, *Xenopus*, mouse and human *Nodal* genes, mouse and human *Lefty2* genes, and mouse and *Xenopus Pitx2* genes (Saijoh et al., 2000; Osada et al., 2000; Yashiro et al., 2000; Shiratori et al., 2001). Our findings add *cCer* to this list, and underscore the essential role of evolutionarily conserved FoxH1-SMAD modules in the transcriptional regulation of asymmetric gene expression (Osada et al., 2000).

Although chick *Cer*, zebrafish *charon* and mouse *Cerl-2* have different expression patterns, the Cerberus-related proteins encoded by these genes seem to have a conserved function in left-right development, which is to restrict Nodal signaling to the left side of the embryo. *Nodal* expression in the node region also differs among vertebrate species: it is bilateral in zebrafish and in early mouse embryos, whereas it is restricted to the left side in the chick embryo (reviewed in Raya and Izpisua-Belmonte, 2004). This difference may justify the need for a Nodal antagonist in the node of zebrafish and mouse embryos, which is not required in the chick node.

The divergence in gene regulation between chick, *Xenopus* and mouse *Cerberus* homologues, here demonstrated by the presence of a FoxH1-SMAD module in the *cCer* regulatory region, has conveyed a novel scenery for the activity of cCer and enabled it to take the role of Lefty2 as a negative-feedback regulator of Nodal signaling in the left lateral plate. In the future, the analysis of novel Cerberus-related molecules involved in the left-right development of other vertebrate species (such as *Xenopus* and rabbit) should provide further insight into the evolution of *Cerberus* gene regulation and function.

We are grateful to J. C. Izpisúa Belmonte, H. Hamada, D. Henrique, S. Piccolo and C. D. Stern for probes and plasmids; S. Marques, N. Moreno, I. Marques and B. Lenhart for technical assistance; and V. Teixeira, A. Jacinto and L. Saúde for critically reading the manuscript. This work was supported by Fundação para a Ciência e a Tecnologia (POCTI/CBO/46691/2002, POCTI/BME/46257/2002 and POCI/SAU-MMO/59725/2004), Centro de Biologia do Desenvolvimento and IGC/Fundação Calouste Gulbenkian, where J.A.B. is a Principal Investigator.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/11/2051/DC1

References

Belo, J. A., Bouwmeester, T., Leyns, L., Kertesz, N., Gallo, M., Follettie, M. and De Robertis, E. M. (1997). Cerberus-like is a secreted factor with neutralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech. Dev.* **68**, 45-57.

Belo, J. A., Bachiller, D., Agius, E., Kemp, C., Borges, A. C., Marques, S., Piccolo, S. and De Robertis, E. M. (2000). Cerberus-like is a secreted BMP and Nodal antagonist not essential for mouse development. *Genesis* **26**, 265-270.

Bertocchini, F. and Stern, C. D. (2002). The hypoblast of the chick embryo positions the primitive streak by antagonizing nodal signaling. *Dev. Cell* **3**, 735-744.

Biben, C., Stanley, E., Fabri, L., Kotecha, S., Rhinn, M., Drinkwater, C., Lah, M., Wang, C. C., Nash, A., Hilton, D. et al. (1998). Murine Cerberus homologue mCer-1: a candidate anterior patterning molecule. *Dev. Biol.* **194**, 135-151.

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

Brennan, J., Lu, C. C., Norris, D. P., Rodriguez, T. A., Beddington, R. S. and Robertson, E. J. (2001). Nodal signaling in the epiblast patterns the early mouse embryo. *Nature* **411**, 965-969.

Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A. and Tsien, R. Y. (2002). A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 7877-7882.

Constam, D. B. and Robertson, E. J. (2000). SPC4/PACE4 regulates a TGFbeta signaling network during axis formation. *Genes Dev.* **14**, 1146-1155.

Faure, S., de Santa Barbara, P., Roberts, D. J. and Whitman, M. (2002). Endogenous patterns of BMP signaling during early chick development. *Dev. Biol.* **244**, 44-65.

Foley, A. C., Skromne, I. S. and Stern, C. D. (2000). Reconciling different models of forebrain induction and patterning: a dual role for the hypoblast. *Development* **127**, 3839-3854.

Hamada, H., Meno, C., Watanabe, D. and Saijoh, Y. (2002). Establishment of vertebrate left-right asymmetry. *Nat. Rev. Genet.* **3**, 103-113.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Hashimoto, H., Rebagliati, M., Ahmad, N., Muraoka, O., Kurokawa, T., Hibi, M. and Suzuki, T. (2004). The Cerberus/Dan-family protein Charon is a negative regulator of Nodal signalling during left-right patterning in zebrafish. *Development* **131**, 1741-1753.

Hsu, D. R., Economides, A. N., Wang, X., Eimon, P. M. and Harland, R. M. (1998). The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Mol. Cell* **1**, 673-683.

Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J. and Hill, C. S. (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol. Pharmacol.* **62**, 65-74.

Ishimaru, Y., Yoshioka, H., Tao, H., Thisse, B., Thisse, C., Wright, C. V. E., Hamada, H., Ohuchi, H. and Noji, S. (2000). Asymmetric expression of antivin/lefty1 in the early chick embryo. *Mech. Dev.* **90**, 115-118.

Juan, H. and Hamada, H. (2001). Roles of nodal-lefty regulatory loops in embryonic patterning of vertebrates. *Genes Cells* **6**, 923-930.

Le Good, J. A., Joubin, K., Giraldez, A. J., Ben-Haim, N., Beck, S., Chen, Y., Schier, A. F. and Constam, D. B. (2005). Nodal stability determines signaling range. *Curr. Biol.* **15**, 31-36.

Liguori, G. L., Echevarria, D., Improta, R., Signore, M., Adamson, E., Martinez, S. and Persico, M. G. (2003). Anterior neural plate regionalization in cripto null mutant mouse embryos in the absence of node and primitive streak. *Dev. Biol.* **264**, 537-549.

Long, S., Ahmad, N. and Rebagliati, M. (2003). The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. *Development* **130**, 2303-2316.

Marques, S., Borges, A. C., Silva, A. C., Freitas, S., Cordenonsi, M. and Belo, J. A. (2004). The activity of the Nodal antagonist Cerl-2 in the mouse node is required for correct L/R body axis. *Genes Dev.* **18**, 2342-2347.

Medina, A., Reintsch, W. and Steinbeisser, H. (2000). Xenopus frizzled 7 can act in canonical and non-canonical Wnt signaling pathways: implications on early patterning and morphogenesis. *Mech. Dev.* **92**, 227-237.
Meno, C., Takeuchi, J., Sakuma, R., Koshiba-Takeuchi, K., Ohishi, S., Saijoh, Y., Miyazaki, J., ten Dijke, P., Ogura, T. and Hamada, H. (2001). Diffusion of nodal signaling activity in the absence of the feedback inhibitor Lefty2. *Dev. Cell* **1**, 127-38.
Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.
Monsoro-Burq, A. H. and Le Douarin, N. M. (2001). Bmp4 plays a key role in left-right patterning in chick embryos by maintaining Sonic Hedgehog asymmetry. *Mol. Cell* **7**, 789-799.
Mostert, V., Sandra, W., Dreher, I., Köhrle, J. and Abel, J. (2001). Identification of an element within the promoter of human selenoprotein P responsive to transforming growth factor-beta. *Eur. J. Biochem.* **268**, 6176-6181.
Nagy, A., Gertsenstein, M., Vintersten, K. and Behringer, R. (2003). *Manipulating the Mouse Embryo: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory Press.
Nakaguchi, E., Mochizuki, A., Yamamoto, M., Yashiro, K., Meno, C. and Hamada, H. (2006). Generation of robust left-right asymmetry in the mouse embryo requires a self-enhancement and lateral-inhibition system. *Dev. Cell* **11**, 495-504.
New, D. A. T. (1955). A new technique for the cultivation of the chick embryo in vitro. *J. Embryol. Exp. Morphol.* **3**, 326-331.
Nieuwkoop, P. D. and Faber J. (1967). *Normal Table of Xenopus laevis (Daudin).* Amsterdam: North-Holland.
Niwa, H., Yamamura, K. and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-200.
Osada, S. I., Saijoh, Y., Frisch, A., Yeo, C. Y., Adachi, H., Watanabe, M., Whitman, M., Hamada, H. and Wright, C. V. (2000). Activin/Nodal responsiveness and asymmetric expression of a Xenopus nodal-related gene converge on a FAST-regulated module in intron 1. *Development* **127**, 2503-2514.
Pagán-Westphal, S. M. and Tabin, C. J. (1998). The transfer of left-right positional information during chick embryogenesis. *Cell* **93**, 25-35.
Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T. and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**, 707-710.
Piedra, M. E. and Ros, M. A. (2002). BMP signaling positively regulates Nodal expression during left right specification in the chick embryo. *Development* **129**, 3431-3440.
Quandt, K., Frech, K., Karas, H., Wingender, E. and Werner, T. (1995). MatInd and MatInspector – New fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* **23**, 4878-4884. (http://www.genomatix.de/).
Raya, A. and Izpisua-Belmonte, J. C. (2004). Unveiling the establishment of left–right asymmetry in the chick embryo. *Mech. Dev.* **121**, 1043-1054.
Rodriguez Esteban, C., Capdevila, J., Economides, A. N., Pascual, J., Ortiz, A. and Izpisua Belmonte, J. C. (1999). The novel Cer-like protein Caronte mediates the establishment of embryonic left-right asymmetry. *Nature* **401**, 243-251.
Saijoh, Y., Adachi, H., Sakuma, R., Yeo, C. Y., Yashiro, K., Watanabe, M., Hashiguchi, H., Mochida, K., Ohishi, S., Kawabata, M. et al. (2000). Left–right asymmetric expression of lefty2 and nodal is induced by a signaling pathway that includes the transcription factor FAST2. *Mol. Cell* **5**, 35-47.
Saijoh, Y., Oki, S., Ohishi, S. and Hamada, H. (2003). Left–right patterning of the mouse lateral plate requires nodal produced in the node. *Dev. Biol.* **256**, 160-172.
Schier, A. (2003). Nodal signaling in vertebrate development. *Annu. Rev. Cell Dev. Biol.* **19**, 589-621.
Schier, A. F. and Shen, M. M. (2000). Nodal signalling in vertebrate development. *Nature* **403**, 385-389.
Schlange, T., Arnold, H.-H. and Brand, T. (2002). BMP2 is a positive regulator of Nodal signaling during left-right axis formation in the chicken embryo. *Development* **129**, 3421-3429.
Shawlot, W., Deng, J. M. and Behringer, R. R. (1998). Expression of the mouse cerberus-related gene, Cerr1, suggests a role in anterior neural induction and somitogenesis. *Proc. Natl. Acad. Sci. USA* **95**, 6198-6203.
Shiratori, H., Sakuma, R., Watanabe, M., Hashiguchi, H., Mochida, K., Sakai, Y., Nishino, J., Saijoh, Y., Whitman, M. and Hamada, H. (2001). Two-step regulation of left–right asymmetric expression of Pitx2: initiation by nodal signaling and maintenance by Nkx2. *Mol. Cell* **7**, 137-149.
Summerton, J., Stein, D., Huang, S. B., Matthews, P., Weller, D. and Partridge, M. (1997). Morpholino and phosphorothioate antisense oligomers compared in cell-free and in-cell systems. *Antisense Nucleic Acid Drug Dev.* **7**, 63-70.
Waldrip, W. R., Bikoff, E. K., Hoodless, P. A., Wrana, J. L. and Robertson, E. J. (1998). Smad2 signalling in extraembryonic tissues determines anterior-posterior polarity of the early mouse embryo. *Cell* **92**, 797-808.
Yamamoto, M., Mine, N., Mochida, K., Sakai, Y., Saijoh, Y., Meno, C. and Hamada, H. (2003). Nodal signaling induces the midline barrier by activating Nodal expression in the lateral plate. *Development* **130**, 1795-1804.
Yashiro, K., Saijoh, Y., Sakuma, R., Tada, M., Tomita, N., Amano, K., Matsuda, Y., Monden, M., Okada, S. and Hamada, H. (2000). Distinct transcriptional regulation and phylogenetic diversity of human LEFTY genes. *Genes Cells* **5**, 343-357.
Yee, S. P. and Rigby, P. W. (1993). The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes Dev.* **7**, 1277-1289.
Yokouchi, Y., Vogan, K. J., Pearse, R. V., 2nd and Tabin, C. J. (1999). Antagonistic signaling by Caronte, a novel Cerberus-related gene, establishes left-right asymmetric gene expression. *Cell* **98**, 573-583.
Yoshioka, H., Meno, C., Koshiba, K., Sugihara, M., Itoh, H., Ishimaru, Y., Inoue, T., Ohuchi, H., Semina, E. V., Murray, J. C. et al. (1998). Pitx2, a bicoid type homeobox gene, is involved in a Lefty-signaling pathway in determination of left–right asymmetry. *Cell* **94**, 299-305.
Zhou, S., Zawel, L., Lengauer, C., Kinzler, K. W. and Vogelstein, B. (1998). Characterization of human FAST-1, a TGFbeta and activin signal transducer. *Mol. Cell* **2**, 121-127.
Zhu, L., Marvin, M. J., Gardiner, A., Lassar, A. B., Mercola, M., Stern, C. D. and Levin, M. (1999). Cerberus regulates left-right asymmetry of the embryonic head and heart. *Curr. Biol.* **9**, 931-938.