

Cerberus regulates left–right asymmetry of the embryonic head and heart

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Background: Most of the molecules known to regulate left–right asymmetry in vertebrate embryos are expressed on the left side of the future trunk region of the embryo. Members of the protein family comprising Cerberus and the putative tumour suppressor Dan have not before been implicated in left–right asymmetry. In *Xenopus*, these proteins have been shown to antagonise members of the transforming growth factor β (TGF- β) and Wnt families of signalling proteins.

Results: Chick Cerberus (*cCer*) was found to be expressed in the left head mesenchyme and in the left flank of the embryo. Expression on the left side of the head was controlled by Sonic hedgehog (Shh) acting through the TGF- β family member Nodal; in the flank, *cCer* was also regulated by Shh, but independently of Nodal. Surprisingly, although no known targets of Cerberus are expressed asymmetrically on the right side of the embryo at these stages, misexpression of *cCer* on this side of the embryo led to upregulation of the transcription factor *Pitx2* and reversal of the direction of heart and head turning, apparently as independent events. Consistent with the possibility that *cCer* may be acting on bilaterally expressed TGF- β family members such as the bone morphogenetic proteins (BMPs), this result was mimicked by right-sided misexpression of the BMP antagonist, Noggin.

Conclusions: Our findings suggest that *cCer* maintains a delicate balance of different TGF- β family members involved in laterality decisions, and reveal the existence of partially overlapping molecular pathways regulating left–right asymmetry in the head and trunk of the embryo.

Background

Although the basic body plan of vertebrates is essentially bilateral, several aspects show consistent left–right asymmetry. The earliest gross morphological asymmetry to develop is rotation of the heart tube, which loops to the right. Very soon afterwards, in higher vertebrates, a twisting of the body occurs, which starts in the head by dorsal exposure of the right eye and ear and gradually travels caudally along the trunk. On the basis of microsurgical experiments, Waddington [1] proposed that the direction of head turning is controlled by the asymmetry of heart looping.

Until recently, the molecular basis of left–right asymmetry remained obscure. In the last few years, however, a complex cascade of events has started to be uncovered, involving secreted molecules and their receptors, cytoskeletal and extracellular matrix components, gap junctions and transcription factors (reviewed in [2–10]). Among these molecules, the secreted factors are particularly interesting because they point to the existence of non-cell-autonomous inductive events that generate or refine laterality. The secreted factors implicated in left–right asymmetry in all vertebrates studied so far are:

Activin, Vg1, bone morphogenetic protein 4 (BMP4), Nodal, Lefty-1 and Lefty-2, which are members of the transforming growth factor β (TGF- β) superfamily, Follistatin, an inhibitor of some of these TGF- β family members, Sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF8) [11–16]. A hierarchy of interactions is starting to be established among the growth and transcription factors: Activin/Vg1-related activities control *Shh* expression, Shh protein then regulates *nodal/lefty* expression, Nodal and Lefty proteins in turn regulate expression of the transcription factor *Pitx2* [17–23] and, subsequently, several aspects of laterality, such as turning of the heart and gut rotation, independently [2,8,11,12].

Here, we report that the chick *Cerberus* (*cCer*) gene, which is related to *Xenopus Cerberus* (*Xcer*) [24] and to mouse *mCer-1/mCer-1* [25,26], is expressed in the left lateral plate mesoderm and left head mesenchyme of the embryo. The asymmetric expression of *cCer* was found to be controlled by Shh in both the head and trunk. In the head, this control was effected through Nodal; in the trunk, Nodal was not involved. Misexpression of *cCer* on the right side of the embryo induced *Pitx2* expression

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and caused reversal of head and heart turning. The endogenous domain of *cCer* expression is adjacent to and overlapping with that of *nodal* and the induction of *Pitx2* on the right side of the embryo by *cCer* could be mimicked by misexpressing the BMP antagonist Noggin. On the basis of these results and previously reported activities of members of the Cerberus family, we propose that *cCer* acts as a subtle regulator of the activity of Nodal/BMP pathways during the establishment of bilateral asymmetry.

Results

Isolation of a chick homologue of *Cerberus*

A *cCer* cDNA was cloned by PCR using chick embryonic stage 4 cDNA as a template and two degenerate primers corresponding to regions conserved between *Xcer* [24] and a mouse expressed sequence tag fragment (EST clone AA120122) representing *mCer-1/mCer-1* [25,26]. A 188 bp fragment was amplified, which had significant similarity to *Xcer*, and was used to screen a chick embryonic stage 5–9 cDNA library [27]. Seven clones with identical open reading frames were obtained.

The open reading frame encoded a 272 amino-acid protein with significant similarity to both *Xenopus* (31% identity overall; [24]) and mouse (44% identity overall; [25,26]) Cerberus family members (Figure 1). In the more highly conserved cysteine-knot region, *cCer* was 65% identical to *mCer-1* and 61% identical to *Xcer*, but only 34% identical in

this region to *Xenopus gremlin* (*Xgrem*), which is a more distant Cerberus family member.

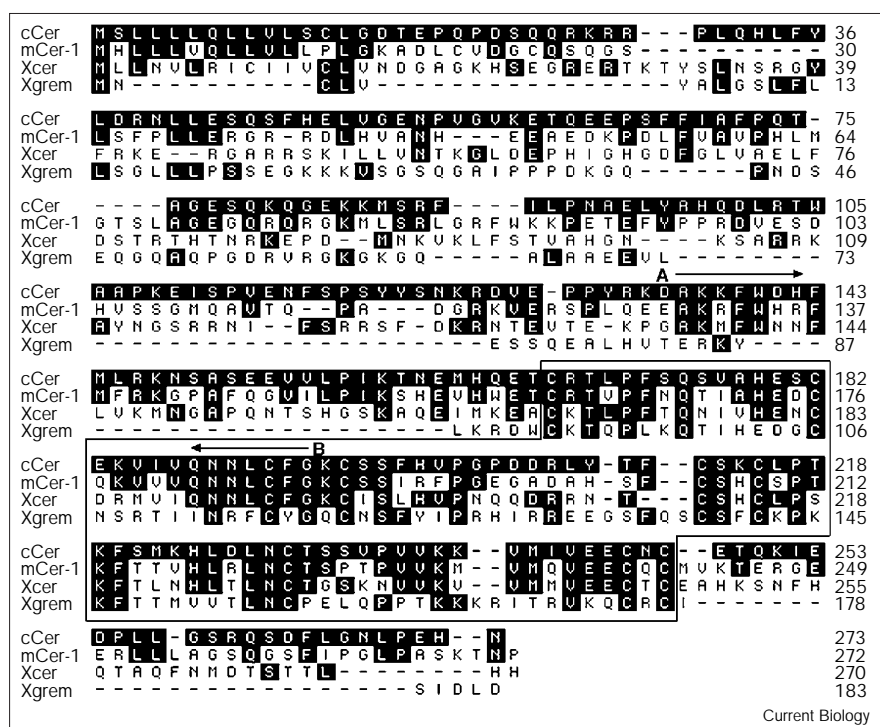
Asymmetric expression of *cCer* during neurulation

At embryonic stage 8–10 [28], *cCer* is expressed in a striking pattern, appearing as a vertical stripe along the length of the left lateral plate mesoderm of the flank (Figure 2a,b). At stage 8, a medial extension of this domain is seen at the level of the second prospective somite (Figure 2a). As more somites form, the expression domain gradually expands caudally (Figure 2b). The lateral expression domain extends rostrally into the head mesenchyme. At this stage, the head mesenchyme shows bilateral expression of *cCer* (Figure 2a) but, by stage 9, all expression on the right side has disappeared (Figure 2b). Histological analysis revealed that the expression of *cCer* is mesodermal (Figure 2d,e). The expression of *cCer* is reminiscent of that of *nodal*, which is also expressed along the left flank and also extends into the left side of the head at stages 8–9 (see Figure 2i in [17]). Double *in situ* hybridisation with *nodal* and *cCer* probes revealed that the two genes overlap in expression except in the most medial domain (prospective somite), where *nodal* expression is found just caudal to the somitic expression of *cCer* (Figure 2c–e).

Misexpression of *Shh* on the right side induces ectopic *cCer* expression

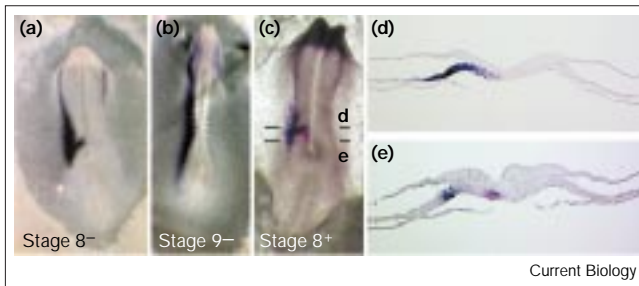
Shh is expressed on the left side of Hensen's node at embryonic stage 5 and is responsible for the induction of

Figure 1



Alignment of the amino-acid sequence of *cCer* with those of *mCer-1/mCer-1*, *Xcer* and *Xenopus Gremlin* (*Xgrem*). The numbers on the right indicate the positions of amino-acid residues, and identical residues have been shaded. A and B are the amino-acid residues corresponding to the PCR primers used to isolate the initial cDNA fragment. The box outlines the cysteine-knot region. The sequence of *cCer* has been deposited in GenBank and assigned accession number AF139721.

Figure 2



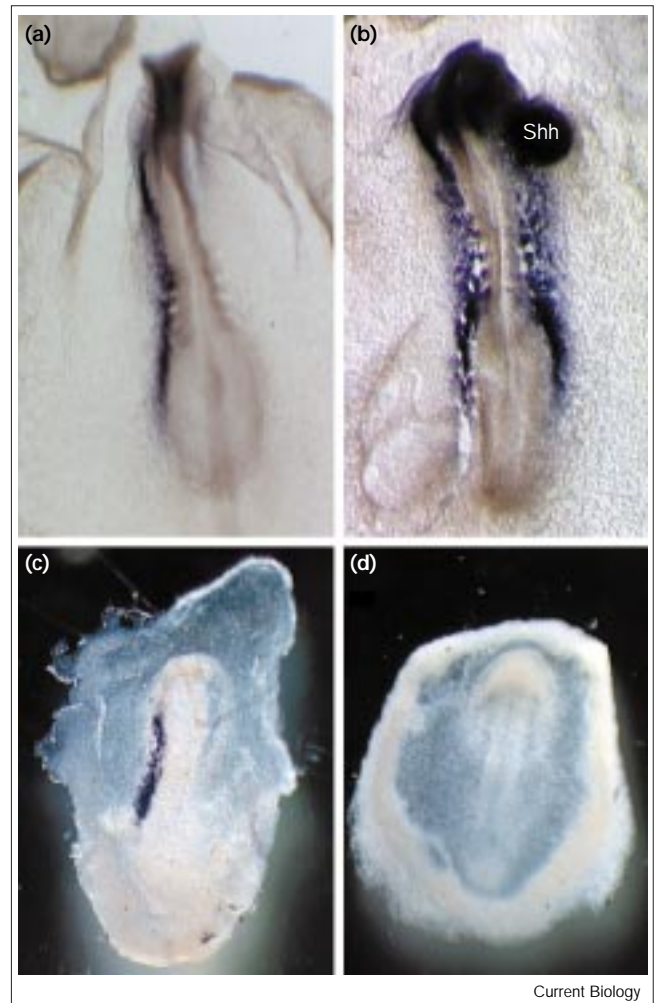
Expression of *cCer* at embryonic stages 8–9. (a) At stage 8⁻, *cCer* is expressed in the left lateral plate mesoderm, bilaterally in the head and in a small horizontal domain coincident with the second caudal somite. (b) By stage 9⁻, expression in the right side of the head has disappeared and all remaining expression in both the head and trunk is on the left. The most medial domain of expression expands caudally as new somites form. (c) Two-probe *in situ* hybridisation for *cCer* (blue/purple) and *nodal* (magenta) in an embryo at stage 8⁺. Note that the *nodal* expression domain in the somitic mesoderm lies slightly caudal to the *cCer*-expressing cells in prospective somite 3. More laterally, *nodal* and *cCer* expression overlap. (d,e) Transverse sections at the levels indicated by the lines in (c) revealed that *cCer* expression is restricted to the mesoderm.

nodal, which is expressed in the left lateral plate mesoderm at stages 7–8 [11,29]. To determine whether the left-sided expression of *cCer* is the result of Shh signalling, we implanted cell pellets expressing Shh on the right side of Hensen's node at stage 4⁺. Embryos were allowed to grow to stage 8 and *cCer* expression detected by *in situ* hybridisation. In control experiments — implants of cells infected with a virus encoding alkaline phosphatase — only normal left-sided *cCer* expression was observed (Figure 3a, *n* = 11). In contrast, embryos implanted with Shh-expressing cell pellets showed ectopic right-sided *cCer* expression in 27% of the cases (Figure 3b, *n* = 30). In all cases, the domain of ectopic expression was very similar to the normal contralateral expression domain. Therefore, misexpression of Shh on the right side of the embryo induced ectopic *cCer* expression in a domain that resembled its endogenous pattern, but on the right side of the embryo.

Shh signalling is necessary for left-sided expression of *cCer*

These results led us to ask whether the endogenous left-sided *cCer* expression domain requires induction by Shh. We investigated this possibility using anti-Shh antibodies that block Shh activity in chick embryos [29,30]. Embryos cultured on floating filters displayed the normal left-sided expression pattern of *cCer* (Figure 3c, *n* = 23). In contrast, in the presence of the anti-Shh antibody, expression of *cCer* was abolished in 81% of the embryos (Figure 3d, *n* = 16). We conclude that Shh signalling is necessary for the normal pattern of expression of *cCer*.

Figure 3

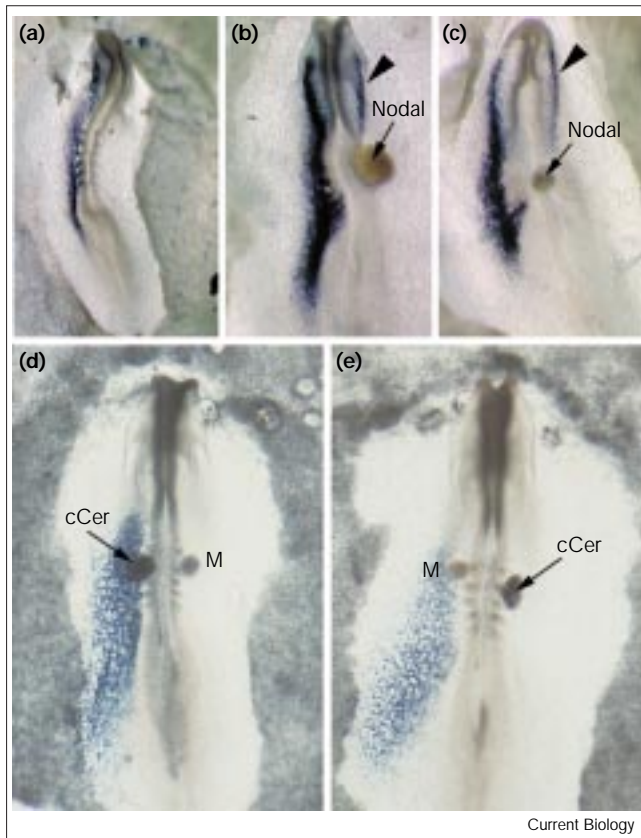


Shh controls *cCer* expression. (a) A pellet of cells infected with a virus expressing alkaline phosphatase (control) was implanted on the right; no change was seen in *cCer* expression. (b) Pellets of cells infected with a Shh-expressing virus, implanted on the right induced ectopic expression of *cCer* on the right side. (c) Embryos cultured on floating filters always displayed the normal left-sided *cCer* expression. (d) In the presence of Shh-blocking antibody, *cCer* expression was lost.

Misexpression of Nodal on the right side induces ectopic *cCer* expression in the head but not in the trunk

Asymmetric expression of *Shh* is seen most strongly at stage 5, well before *cCer* expression; the previous experiments also suggest that Shh signalling is upstream of *cCer*. The *nodal* gene is, however, first expressed asymmetrically on the left side of Hensen's node at stage 7, just before the onset of the asymmetric expression of *cCer*. To determine whether *nodal* is also upstream of *cCer*, cell pellets expressing Nodal protein were implanted on the right side of Hensen's node at stage 6⁺, the embryos allowed to grow to stage 8 and *cCer* expression detected by *in situ* hybridisation. Interestingly, Nodal did not induce ectopic *cCer* expression in the right flank (*n* = 14;

Figure 4



Misexpression of Nodal on the right of the embryo induces ectopic *cCer* expression in the head but not in the flank. (a) Normal expression of *cCer* (purple stain) in the left lateral plate mesoderm and on the left side of the head. (b) When cells expressing Nodal were implanted into the right side (arrow), ectopic *cCer* expression was observed on the right side in the head (arrowhead) but not in the lateral plate mesoderm. (c) This effect was seen even when the pellet was grafted in a caudal position. (d,e) By contrast, misexpression of *cCer* did not alter *nodal* expression (blue stain), regardless of whether the *cCer* pellet was placed (d) on the left or (e) on the right: *cCer*, pellet of *cCer*-expressing cells; M, pellet of mock-transfected (control) cells.

Figure 4b,c) but did induce ectopic right-sided expression of *cCer* in the head in 43% of the embryos ($n = 14$; Figure 4b,c). This was the case even when the Nodal pellet was placed within the right flank: ectopic expression of *cCer* was still only seen in the head, which is some distance from the graft (Figure 4c). These results suggest that the two domains of *cCer* expression, in the head and in the lateral plate mesoderm, are regulated by different mechanisms. Whereas *Shh* regulates both domains, Nodal only regulates *cCer* expression in the head.

To investigate the possibility of a feedback regulatory loop between *cCer* and *nodal*, mock-transfected cells or cells expressing *cCer* were implanted in the lateral plate at stage 7–8 and the embryos cultured for 6–8 hours (to stage 9). No effect on *nodal* expression was seen whether

the graft was placed on the right or on the left ($n = 11$ and 9, respectively; Figure 4d,e). Together, these results allow us to conclude that *nodal* lies upstream of *cCer* in the head. This genetic hierarchy is similar to that which has been found for dorsoventral and rostrocaudal patterning in *Xenopus*: Nodal induces Cerberus, which in turn antagonises Nodal activity [31–33].

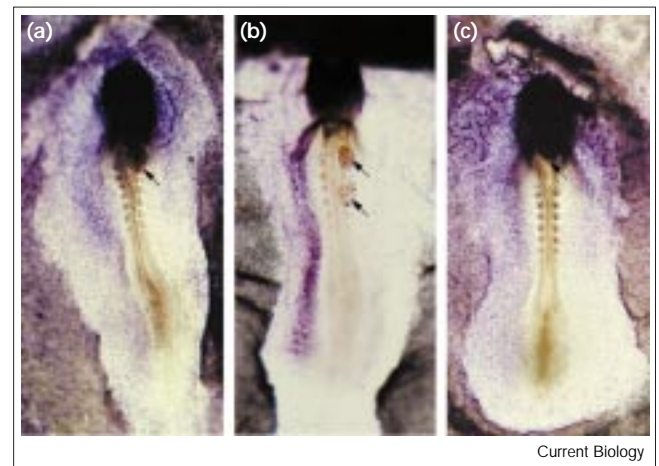
Pitx2 is induced by *cCer*

Pitx2 encodes a transcription factor that is normally expressed in the left flank but bilaterally in the head [17–23]. Therefore, we investigated whether *cCer* can regulate *Pitx2* expression in the head and/or trunk. A single 1,000-cell pellet of cells expressing *cCer* implanted on the right side had no effect on *Pitx2* expression ($n = 7$). Two pellets, however, induced a broad ectopic domain of *Pitx2* expression in the right flank, albeit weaker than the endogenous expression domain. When the pellet was placed close to the head, ectopic expression extended to the normally non-expressing region surrounding the head (Figure 5a; $n = 15$). These findings are consistent with previous proposals that *Pitx2* encodes a downstream effector of left–right asymmetry [17–23].

cCer regulates the direction of head turning and heart polarity

To test directly the role of *cCer* in the development of left–right asymmetry, cells expressing *cCer* were implanted on the right side of the node or right lateral plate mesoderm at stage 4–9. After incubation of the embryos to

Figure 5



Induction of *Pitx2* expression by *cCer* and Noggin. (a) When two pellets of *cCer*-expressing cells (arrow) were implanted on the right side, a broad ectopic domain of *Pitx2* expression (purple stain) appeared along the right flank of the embryo. Expression extended into regions surrounding the head. (b,c) Effect of BMP antagonists. (b) Misexpression of Chordin (arrows) on the right side had no effect on *Pitx2* expression, whereas (c) misexpression of Noggin (arrow) on the right side induced an ectopic domain of *Pitx2* expression.

Table 1

Effects of cCer misexpression in the lateral plate mesoderm.

| Embryonic stage at which cells were implanted | cCer-expressing cells implanted on the right side | | Mock-transfected cells implanted on the right side | | cCer-expressing cells implanted on the left side | | Mock-transfected cells implanted on the left side | |
|---|---|------|--|------|--|------|---|------|
| | Heart | Head | Heart | Head | Heart | Head | Heart | Head |
| Stage 4–5 | 1/13 | 1/10 | 1/9 | 1/6 | 1/12 | 1/5 | 0/12 | 0/7 |
| Stage 6–7 | 10/35 | 9/33 | 0/26 | 0/23 | 1/17 | 1/15 | 0/15 | 0/11 |
| Stage 8–9 | 2/16 | 2/14 | 0/8 | 0/8 | 2/15 | 2/13 | 1/15 | 1/13 |

The numbers of embryos with a reversed heart or a reversed head, out of the total number of embryos surviving to an appropriate stage, are shown.

stage 12–14, the direction of heart looping and head turning was scored. When cCer was misexpressed on the right side of stage 6–7 embryos, 10/35 embryos (28%) showed inverted heart looping, whereas none of the 26 embryos that had received mock-transfected cells on the right side showed reversed looping (Table 1). Of the 10 embryos with reversed hearts, 9 survived to later stages and the heads of all of them had turned to the left side (normally to the right). Mock-transfected cells ($n = 42$) and cells expressing cCer ($n = 44$) were also implanted on the left side of embryos. In this case, no difference in heart looping or head turning was observed between the two sets at any of the stages tested (Table 1). Interestingly, misexpression of cCer on the right at earlier (stage 4–5) or later (stage 8–9) stages had little or no effect on the direction of heart looping or head turning compared with mock-transfected controls (Table 1). These results suggest that there is a narrow time window during which cCer can regulate left–right polarity.

Does cCer act on the heart and the head to control their polarity independently, or is its primary action on only one of these systems? Both possibilities are likely for several reasons. First, because we have found on the one hand that cCer misexpression caused defects in head turning in all cases where heart looping was also affected, and on the other hand that misexpression of Nodal on the right side of the embryo induced the expression of *cCer* in the head but not the flank. Second, *Pitx2* is expressed on the left side in the flank but symmetrically in the head [17,22]. Finally, it has been proposed that the heart situs can mechanically affect the asymmetry of head turning [1]. To answer the question, we implanted mock-transfected cells or cCer-expressing cells into the right side of the head just beneath the head ectoderm of stage 9–10 embryos. In the control group, all 43 embryos had normal head and heart polarities. In 5/41 embryos that received cCer-expressing cells, head polarity was affected (4 showed reversed direction of head turning and 1 showed no turning), but the direction of heart looping was only affected in 3 of these 5 (Table 2). Despite the small number of observations, this

experiment suggests that misexpression of cCer can redirect the turning of the head even in embryos where the asymmetry of heart looping is normal.

Right-sided misexpression of Noggin mimics misexpression of cCer

Cerberus has been reported to be an antagonist of several TGF- β family members, including Nodal; this led us to expect that the effects of Cerberus might be seen upon overexpression on the left side of the embryo, where Nodal is present. The finding that cCer misexpression was only effective on the right side raised the possibility that its targets may be other TGF- β family members. At this stage of development, BMPs 2, 4 and 7 are expressed bilaterally, at the edge of the neural plate and in the lateral mesoderm [34–36]. We tested the possibility that the targets of cCer on the right side of the embryo are BMPs by misexpressing Chordin or Noggin on the right side. Even when two pellets of Chordin-expressing cells were implanted, neither the expression of *Pitx2* nor the polarity of heart or head turning was affected (Figure 5b; $n = 7$). Noggin, on the other hand, induced *Pitx2* expression (Figure 5c; $n = 6$) and reversed heart looping in 2/3 cases. These results are consistent with the possibility that BMPs are targets of cCer action.

Table 2

Effects of cCer misexpression on the right side of the head.

| | | cCer-expressing cells implanted | Mock-transfected cells implanted |
|-------|-------------------|---------------------------------|----------------------------------|
| | | Head | Reversed polarity |
| | No turning | 1/41 | 0/43 |
| Heart | Reversed polarity | 3/41 | 0/43 |

The numbers of embryos with a reversed heart or a reversed head, out of the total number of embryos surviving to an appropriate stage, are shown.

Discussion

Cerberus, together with Xgremlin and Dan, define a family of molecules (the Dan family) that have been implicated in dorsoventral patterning, neural induction and head specification in *Xenopus*. These molecules act as secreted inhibitors of members of the TGF- β family (BMPs, Nodal and, to some extent, Activin) as well as of members of the Wnt family [31–33]. Here, we report that *cCer*, which shares significant structural homology to *Xcer* and to mouse *mCer-1*, has a striking pattern of expression during neurulation in the chick embryo; expression was found to be restricted to the left lateral plate mesoderm and head mesenchyme. We also found that misexpression of *cCer* on the right side of the trunk induced *Pitx2* expression and affected the polarity of both the head and heart; misexpression on the right of the head appeared to affect the direction of head turning independently of a prior effect on heart polarity. The left-sided expression of *cCer* was found to be regulated by Shh, both in the head and in the trunk; expression on the left side in the head, but not in the trunk, was also controlled by Nodal.

Our results suggest that *cCer* is a secreted regulator of left–right asymmetry, situated downstream of the previously reported signals Shh and Nodal. They also reveal a split in the molecular pathways that set up laterality in the head and trunk. In the trunk, *cCer* is controlled by Shh activity independently of Nodal, whereas in the head it is controlled by Shh through Nodal. The initial expression of *cCer* was found to be bilateral at stage 8, the stage at which *Pitx2* is first expressed, also bilaterally, in the head. These observations, together with the finding that *cCer* induced *Pitx2*, suggest that *cCer* might be responsible for initiating bilateral expression of *Pitx2* in the head. A direct test of this has to await loss-of-function experiments for *cCer* or its orthologue in other species. In mice, this orthologue might be the gene *Dte*, which appears to be the only member of the family with asymmetric expression [37].

How does *cCer* act to regulate left–right polarity? *Xcer* appears to be unique among the Dan family members in being able to block Nodal-like and Activin-like activities, whereas all members of the family block BMP2 [31]. The site and timing of asymmetric *cCer* expression in the chick suggest that Nodal is a likely target for inhibition, as it is expressed directly adjacent to and partly overlapping with the *cCer* expression domain. Because all of the TGF- β superfamily members with asymmetric distribution at stages 6–9 that have been described to date are expressed on the left side, we were surprised to find that misexpression of *cCer* on the right, rather than the left, led to reversal of left–right polarity of the embryo. Inhibition of cWnt8C activity (which is expressed in a small domain to the right of the node at stage 6–8 [10]) is unlikely to account for the consequences of *cCer* misexpression, because *cCer* pellets implanted on the right side of the

head, far from the small cWnt8C expression domain, were almost as effective as pellets placed in the trunk (Table 2).

As no putative targets of *cCer*/Cerberus are known to be expressed on the right side at the appropriate stage, we tested the possibility that the targets of *cCer* misexpression on the right are TGF- β family members that are expressed in a bilaterally symmetrical way. The most obvious candidates are BMP2, expressed in the lateral mesendoderm, and BMP4 and BMP7, expressed at the edges of the neural plate [34–36]. We found that Noggin, but not Chordin, could mimic the effects of *cCer* misexpression on the right side. The difference in activity between these two BMP antagonists is reminiscent of previous findings that Noggin but not Chordin can induce somites and that Chordin but not Noggin can induce a primitive streak [38], and provides an assay for future identification of the endogenous targets of the BMP antagonists in each of these developmental processes.

On the basis of the results presented here on the expression, regulation and activity of *cCer*, and on available data on the expression of TGF- β and Wnt family members, we propose that, during normal development, *cCer* may function on the left side in part by regulating Nodal activity. Its roles may also include modulation of the activity of BMP2, BMP4 and/or BMP7, which are normally expressed symmetrically at this stage. Taken together, our findings suggest that the regulation of left–right asymmetry requires a subtle balance of the activity of different secreted factors on each side of the midline of the early embryo. We have also provided evidence that head polarity may be controlled in part by the asymmetric expression of *nodal* and *cCer*; contrary to expectations from classical experiments, the direction of head turning can be dissociated experimentally from the polarity of the heart.

Materials and methods

Cloning of *cCer*

A 188 bp fragment homologous to *Xenopus Cerberus* was amplified by PCR using the following two degenerate primers: A, 5'-CA(CT)TTI-CC(AG)AA(AG)CAIA(AG)(AG)TT(AG)TT(CT)TG-3'; and B, 5'-GAIGAI-GC(N)AA(AG)(AC)G(N)TT(CT)TGG-3'. Poly(dT)-primed cDNA was synthesised from stage 4 chick mRNA, and PCR carried out using Taq Polymerase (Boehringer Mannheim) under the following conditions: 3 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 37°C, slow ramp to 72°C at 1°C/sec, 1 min 30 sec at 72°C, with a final extension at 72°C for 15 min. PCR products of the expected size were gel purified and ligated into the *EcoRV* site in pBluescript KS+ (Stratagene). The 188 bp cDNA fragment was used to screen a stage 5–9 Lambda Zap II cDNA library [27]. Sequencing revealed an open reading frame of 816 bases with significant homology to *Xenopus Cerberus* and mouse *mCer-1/mCer-l*. Sequencing from an internal primer in this open reading frame showed that the seven clones isolated all contained identical sequences in the *Cerberus*-related gene, but flanking sequences differed, suggesting that the phage contained tandem inserts. The open reading frame of the chick *Cerberus* gene was amplified from a library insert by PCR using the following primers: 5'-ACAGCATCGGTCT-CACCATGTCACTGCTCCTCCTT-3' (upstream primer) and 5'-ACC-TTGCTGCAGTTAATTGTGCTCAGGTAGAT-3' (downstream primer).

The product was cut with *Bsa*I and *Pst*I and ligated into the *Nco*I and *Pst*I sites of SLAX-13 [39]. To generate a suitable construct for expression studies, the coding region of *cCer* with a sequence encoding the haemagglutinin (HA) epitope tag was released from the plasmid and inserted into the expression vector pcDNA3.1(-)/Myc-His A (Invitrogen). As the *cCer* stop codon was placed before the Myc-His sequence, the expressed protein is tagged only with HA. We used this tag to confirm the expression and secretion of *cCer* protein from transfected COS cells in western blots (data not shown).

In situ hybridisation

In situ hybridisation was done with digoxigenin-labelled riboprobes as described previously [11,40].

Misexpression of Shh and Nodal

These factors were misexpressed by transplanting chick embryo fibroblasts (line 0) infected with an RCAS retrovirus encoding Shh or Nodal (as described previously [11]), or alkaline phosphatase as a control. The cells were passaged several times, and checked by *in situ* hybridisation for gene expression. Cell pellets were made from confluent dishes of cells, and implanted into embryos in paper-ring culture [41]. Embryos (staged according to Hamburger and Hamilton [28]) were then cultured on albumen until stage 8, fixed in 4% paraformaldehyde, and processed for *in situ* hybridisation; some embryos were then embedded in plastic and sectioned.

Shh antibody experiments

Embryos at stage 4 were detached from their membranes, and the area opaca trimmed. The embryos were then laid flat, ectoderm downwards, onto polycarbonate filters (Corning, 1 AA0044DE52), which were floated over 2 ml medium (DMEM with 5% egg albumen), and cultured at 38°C in 5% CO₂ until stage 8. The medium for experimental embryos also contained approximately 100 µg/ml anti-Shh antibody (5E1). The antibody, developed by T.M. Jessell, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, and the Department of Biological Sciences, University of Iowa, Iowa City, under contract NO1-HD-2-3144 from NICHD.

Misexpression of cCer, Chordin and Noggin

Initial experiments were performed by implanting pellets of chick embryo fibroblasts (CEFs) infected with a replication-competent RCAS retrovirus containing the *cCer* gene, using CEFs infected with RCAS encoding alkaline phosphatase (AP) as a control. Both *cCer*-RCAS and *AP*-RCAS CEFs gave positive results in other assays (they both induced *Sox3* expression when implanted into the area opaca of stage 3+ embryos). We therefore chose to use COS cells transfected with *cCer* in the expression vector pCDNA3.1 instead, and mock-transfected COS cells as a control. COS cells were transfected using Lipofectamine (Gibco-BRL) as previously described [42]. After incubation for 24 h, 1000-cell pellets were prepared as hanging drops. The next day, the pellets were collected and grafted into chick embryos. Successful expression was confirmed by staining the grafted embryos with anti-HA antibody. Cell pellets from *cCer*-expressing or mock-transfected COS cells were grafted into embryos in New culture [43,44]. COS cells expressing Chordin and Noggin were prepared by the same method [36,38,42].

A small slit was made in the ectoderm and one or two cell pellets inserted between the ectoderm and endoderm. The embryos were then cultured to stage 9–10 to assess the expression of *Pitx2*, or to stage 12–15 for scoring the directions of heart looping and head turning. To implant a cell pellet directly into the head region, embryos were accessed *in ovo* as described previously [45]. A cut was made in the head ectoderm at the hindbrain level of stage 9–10 embryos and the pellet pushed into the pocket formed. The eggs were sealed and returned to the incubator for another 24 h before scoring the direction of head turning.

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