Generating asymmetries in the early vertebrate embryo: the role of the Cerberus-like family

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ABSTRACT One fundamental aspect of vertebrate embryonic development is the formation of the body plan. For this process, asymmetries have to be generated during early stages of development along the three main body axes: Anterior-Posterior, Dorso-Ventral and Left-Right. We have been studying the role of a novel class of molecules, the Cerberus/Dan gene family. These are dedicated secreted antagonists of three major signaling pathways: Nodal, BMP and Wnt. Our studies contribute to the current view that the fine tuning of signaling is controlled by a set of inhibitory molecules rather than by activators. In this context, the Cerberus-like molecules emerge as key players in the regulation and generation of asymmetries in the early vertebrate embryo.

KEY WORDS: Cerberus, nodal, gastrulation, body axis, embryogenesis

Introduction

One common feature of all vertebrate embryos is their beginning as a single cell upon fertilization, the fertilized egg or zygote. A subsequent series of mitotic divisions leads to the formation of an apparently unpatterned and uncommitted mass of identical cells. However, subtle differences between these cells arise very early, creating asymmetries within the embryo, which are essential for the development of a complex new organism. Before becoming morphologically visible, the initial embryonic polarities and asymmetries are patent only at the molecular level. Scientists are now starting to unravel the genetic and morphogenetic mechanisms generating polarities in the vertebrate embryo. Ultimately, these processes will lay down the basis for the initial asymmetries that direct the establishment of the three main body axes: the Anterior-Posterior (A-P), Dorso-Ventral (D-V) and Left-Right (L-R) axis.

Many of the initial studies were performed using the amphibian embryo, Xenopus laevis, as an animal model. The basic requirements for the maintenance of the animals, the large amounts of fertilized embryos easily obtained, and the relative large egg size, made them commonly used in embryological studies. In 1924, Hans Spemann and Hilde Mangold demonstrated that transplanted tissue was able to induce the surrounding cells to acquire a new fate, organizing this way the formation of a novel central nervous system (CNS) and axial mesoderm (notochord), and also contributing to the dorsalization of mesoderm and generation of somites. Because of these activities, the dorsal blastopore lip is now also designated as the “Organizer” or “Spemann Organizer”.

With the introduction of novel molecular biology techniques, scientists started to search for the molecular players that hold the inductive activity of the Organizer. Goosecoid was the first Organizer-expressed molecule that was isolated from the Xenopus laevis embryo. Over-expression in the ventral side of the embryo of this homeodomain-containing transcription factor, was able to efficiently generate secondary body axis, thus resembling the activity of the Organizer (Cho et al., 1991). The isolation and study of goosecoid homologous genes in other vertebrate species led to the identification of the organizer as a common feature of the vertebrate embryo. The organizer is known as Hensen’s node in the rabbit and chick, the shield in zebrafish and the anterior end of the primitive streak in the mouse embryo (Cho et al., 1991; Blum et al., 1992; Izpisúa-Belmonte et al., 1993; Stachel et al., 1993; Schulte-Merker et al., 1994). Since these first findings, several other molecules expressed in the vertebrate organizer have been isolated and studied, contributing to its molecular characteriza-
Cerberus/Dan gene family. blast and anterior visceral endoderm, respectively (Bouwmeester et al., 1999). Among the newly identified antagonists, one excelled due to its remarkable properties: *Xenopus cerberus* (*Xcer*). This novel secreted protein is expressed in the most anterior tip of the non-involuting endomesodermal cells located in the deep layer of the Spemann’s organizer (Bouwmeester et al., 1996). Injection of *Xcer* mRNA in ventral blastomeres resulted in the induction of secondary heads. This characteristic phenotype led to its denomination, *Cerberus*, after the mythological dog with three heads that guards the gates of Hades.

*Cerberus*-related proteins have been identified in other vertebrate species (see Table 1): mouse *cerberus*-like gene (*cerl-1*, Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998), chick *Cer* (C.Cer, Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). *Xenopus Coco* (Bell et al., 2003), zebrafish *Charon* (Hashimoto et al., 2004) and mouse *Cerberus*-like-2 (*Cerl-2, Marques et al., 2004), and are now grouped in the *Cerberus/Dan* gene family. *Xenopus Xcer*, chick cCer and mouse *Cerl-1* genes are syntenic (www.metazome.net) and, at peri gastrulation stages, are expressed in topological equivalent embryonic structures, such as the anterior endomesoderm, hypoblast and anterior visceral endoderm, respectively (Bouwmeester et al., 1996; Foley et al., 2000; Belo et al., 1997). In contrast, *Xenopus coco* was found to be expressed during pre-gastrula stages exclusively in the animal pole. At gastrula stages, *coco* transcripts can be detected in both the dorsal and ventral marginal zone as well as in the animal cap ectoderm (Bell et al., 2003). By early neurulation stages, mouse *Cerl-1* and chick cCer transcripts are also detected in the anterior definitive mesendoderm (Belo et al., 1997; Rodriguez-Esteban et al., 1999). But at later stages, during somitogenesis, *Cerberus*-related genes display very distinct expression patterns. *Xcer* expression is no longer observed. Mouse *Cerl-1* transcripts are found in the rostral half of the two newly formed somites and rostral presomitic mesoderm. Chick cCer is expressed in the left paraxial and lateral plate mesoderm. Zebrafish *Charon* and mouse *Cerl-2* are both expressed around the node region but with a remarkable difference between their expression patterns: while *Charon* has a symmetric domain, *Cerl-2* becomes strongly expressed on the right side (Bouwmeester et al., 1996; Belo et al., 1997; Rodriguez-Esteban et al., 1999; Hashimoto et al., 2004; Marques et al., 2004). Very recently, *Xcoco* was shown to be expressed bilaterally in the posterior paraxial mesoderm during neurula stages (Vonica and Brivanlou, 2007).

*Cerberus*-related genes belong to the cysteine-knot superfamily and encode for small secreted proteins (from 185 a.a., *Cerl-2*, to 272 a.a., *Xcer* and cerl-1) with a signal peptide at the N-terminal and a Cystein-Rich Domain (CRD) containing 9 cysteines at the C-terminal region. After secretion these proteins are proteolytically cleaved and form active dimmers (Shawlot et al., 1998; Piccolo et al., 1999). Sequence analysis revealed that these molecules share a significant level of similarity in the CRD but outside of this region, towards the N-terminus, they display very little homology. (Marques et al., 2004; Hashimoto et al., 2004). The spacing of cysteines in the CRD resembles a motif termed the cysteine-knot (McDonald and Hendrickson, 1993; Isaacs, 1995), which is also found in a number of cytokines such as BMP, NGF, PDGF, among others. The CRD of *Cerberus*-related proteins contains the C-X-G-X-C motif, conserved in all cysteine-knot proteins, and 3 of the 4 additional cysteines that are also present in NDP and mucins. This domain is essential for the biological activity of the *Cerberus* molecules, which have been shown to be secreted multivalent antagonists that bind Nodal, BMP and Wnt proteins, and probably inhibit their activity in the extracellular space (Belo et al., 1997; Hsu et al., 1998; Piccolo et al., 1999; Belo et al., 2000; Marques et al., 2004).

**Cerberus in anterior-posterior patterning**

Embryological and genetic studies have provided evidence for the existence of distinct vertebrate head and trunk organizers (Spemann, 1931; Thomas and Beddington, 1996; Belo et al., 1997; Bouwmeester and Leyns, 1997; Schneider and Mercola, 1999). The isolation and study of *Xenopus cerberus* (*Xcer*, Bouwmeester et al., 1996), a novel secreted factor with strong head inducing activity expressed in the anterior dorsal endoderm (ADE), was the first report that pointed to the possible role of this region in the induction of the anterior head. The topographical equivalent of this region in the mouse embryo, the anterior visceral endoderm (AVE), has also been implicated in anterior specification (Thomas and Beddington, 1996). The isolation of a mouse *cerberus*-like gene (*cerl-1*, Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998), expressed in the AVE before and during gastrulation, in a region underlying the prospective anterior neuroectoderm, underscored the inductive role of this region in the mouse embryo.

Microinjection of *Xcer* mRNA in ventral blastomeres results in the induction of secondary heads. Those included fore and midbrain, eye, cement gland and olfactory placodes (Bouwmeester et al., 1996). *Xenopus* animal cap explants are widely used as an assay system for studying cell differentiation induced by microinjection of mRNAs encoding for specific gene products, since uninjected animal caps normally only give rise to epidermis. In animal cap explants, microinjection of *Xcer* mRNA induces anterior central nervous system (CNS) markers such as *Otx2*, but not more posterior ones like *Engrailed-2* or *HoxB9*, consistent with the lack of brain structures posterior to the midbrain in the generated ectopic heads. Endodermal (*endoderm*) and heart (Nkx-2.5) markers are also upregulated in the animal cap experiments (Bouwmeester et al., 1996). In some cases, the induction of those head-like structures was accompanied by duplication of the heart and liver. This fact could be related to the ability of *Xcer* mRNA to induce the referred endodermal and heart markers. *Xcer* is expressed in the yolky cells that form the leading edge of the *Xenopus* gastrulating endoderm. This cell population will eventually give rise to foregut and midgut, including the liver, as observed in cell lineage tracing experiments with Dil (Bouwmeester et al., 1996).

In an effort to determine the genetic and biochemical basis of the head induction by *Xcer*, Piccolo and colleagues demonstrated that *Cerberus* functions as a multivalent growth-factor
Cerberus-like genes and vertebrate patterning

antagonist, binding to Nodal, BMP and Wnt proteins, (Piccolo et al., 1999), thus inhibiting the activity of those proteins in the extracellular space. Using several antagonists of these signaling pathways, it was demonstrated that inhibition of Nodal, BMP and Wnt signals in ventral blastomeres was sufficient to induce a secondary head (Piccolo et al., 1999; Glinka et al., 1997). In view of these facts it was proposed, but not proved, that the secretion of Cerberus into the extracellular space would be necessary to lock the head-organizer programme in place by simultaneously antagonizing three signaling pathways involved in trunk formation, thereby restricting the trunk territory to the posterior part of the body.

In order to try to address this role of XCer, we have assayed a combination of approaches, involving Knock-down, overexpression and tissue recombination experiments. Morpholino oligonucleotides against XCer (Cer-MO) microinjected in the two dorsal-vegetal blastomeres of the 4-8 cell stage Xenopus embryo, impaired the induction of the head, where a reduction in the primary head and eyes could be observed (Silva et al., 2003; Kuroda et al., 2004). To try to uncover the biological roles of XCer and of the ADE in head formation, we decided to challenge the activity and local requirement of Xcerberus during this process. We tackled this by using the mcer-1 promotor to drive expression of BMP, Nodal or Wnt molecules in the ADE. We have previously generated a transgenic mouse line using this promoter element to drive expression of EGFP in the AVE (Mesnard et al., 2004). We also demonstrated that this mouse promoter is specifically activated in the Xenopus ADE and closely resembles the spatiotemporal pattern of expression of endogenous XCer (Silva et al., 2003). In these experiments, targeted increase of BMP, Nodal and Wnt activity (which are proposed to be antagonized by XCer) in the ADE, resulted into a remarkable synergistic loss of anterior head without affecting posterior (trunk) structures. But when we simultaneously depleted XCer levels by co-microinjecting Cer-MO, we observed a strong enhancement of the phenotypes generated by increasing the levels of BMP, Nodal and Wnt activity and the resulting embryos completely lacked the head structures (Silva et al., 2003). These results demonstrated for the first time that in fact, Xcerberus is a biological inhibitor of BMP, Nodal and Wnt signaling and that this activity is required in vivo in the leading edge of the ADE for the proper induction and patterning of the head. This would be accomplished by the generation of a “head-field” protected from posteriorizing trunk signals. Therefore, a correct balance of agonists versus antagonists in the ADE is

TABLE 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Peri-Gastrulation</th>
<th>Post-Gastrulation</th>
<th>Function</th>
<th>References</th>
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<tr>
<td>xcer</td>
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<td>Nodal, Bmp and Wnt antagonist. Head induction and specification. Inhibition of mesoderm signals in the ADE.</td>
<td>Bouwmeester et al., 1996</td>
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<td>mcer-1</td>
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<td>Nodal and Bmp antagonist. Redundant with lefty-1, both genes are required for the correct positioning of the A-P axis by determination of the migration of DVE and restriction of the primitive streak to the posterior region.</td>
<td>Belo et al., 1997</td>
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<td>ccer</td>
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<td>Nodal and Bmp antagonist. Required to prevent the formation of trunk mesoderm in the prospective head neuroectoderm, restricting the localization of the PS. Required to L-R axis specification by restricting Nodal signalling to the left-LPM.</td>
<td>Rodriguez-Esteban et al., 1999</td>
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<tr>
<td>x coco</td>
<td></td>
<td></td>
<td>Bmp, Nodal, Activin, Derrière and Wnt antagonist. Regulates cell fate specification and competence prior to onset of neural induction. Required to restrict TGF-β signalling to the left side of the embryo.</td>
<td>Bell et al., 2003</td>
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<td>z charon</td>
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<td>Nodal antagonist. Required to restrict Nodal signalling to the left side of the embryo.</td>
<td>Hashimoto et al., 2004</td>
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<td>mcer-2</td>
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<td>Nodal and Bmp antagonist. Required in the node to restrict Nodal signaling activation to the left lateral plate mesoderm.</td>
<td>Marques et al., 2004</td>
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crucial for head formation.

Due to this relevant role of Xcer and the ADE in the process of head induction, isolation and study of its mouse homologue would be of major importance for the understanding of this developmental mechanism in the mammalian embryo. We and others have isolated the mouse Cerberus-like gene (cerl-1; Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998). This gene is expressed in the AVE by E5.5 and later is gradually displaced by the emerging anterior definitive endoderm. Animal cap experiments demonstrated that this mouse gene is able to induce the same set of signals as the XCer homologue (Belo et al., 1997; Biben et al., 1998), but is not able to generate the characteristic ectopic heads when microinjected in ventral vegetal blastomeres (Belo et al., 1997). These inductive activities of mCer-1 and Xcer in animal caps are characteristic of inhibition of BMP signaling (Sasai et al., 1996). Using biochemical and functional assays, we demonstrated that in fact cerl-1 binds to and inhibits both BMP and Nodal signals, but unlike its Xenopus counterpart, is not able to bind to Wnt proteins (Belo et al., 2000). Furthermore, tissue recombination experiments demonstrated that the expression of Otx2 was maintained in ectoderm explants recombined with Cerl-1-expressing somitic-presomitic mesoderm (Shawlot et al., 1998).

In summary, cerl-1 is expressed in the AVE, in a region underlying the prospective anterior neuroectoderm, has neural inducing abilities in Xenopus experiments and is able to maintain Otx2 expression in mouse explants.

Otx2 and Lim1 are genes expressed in the AVE and anterior neuroectoderm. Generated KO mouse lines demonstrated their requirement for the proper formation of anterior head structures (Acampora et al., 1995; Matsuo et al., 1995; Shawlot and Behringer, 1995). The AVE is the topological equivalent of the Xenopus ADE and has been implicated in anterior specification (Thomas and Beddington, 1996; Belo et al., 1997). This role was supported by the finding that chimeric mouse embryos composed of a combination of wild-type epiblast, with mutant extraembryonic tissues (namely the AVE) lacking either Otx2 or Lim1, the head is not properly formed (Rhinn et al., 1998; Shawlot et al., 1999). In light of all these properties, cerl-1 was well positioned to be a crucial player of the head organizer programme.

In order to study its role in development, we have generated a targeted inactivation of cerl-1 in the mouse (Belo et al., 2000; Shawlot et al., 2000; Stanley et al., 2000). Surprisingly however, in none of the three generated KO mouse lines an abnormal head or embryonic axis defects were observed, arguing against a previously anticipated essential role of cerl-1 in early development.

Cerl-1 has been demonstrated to have strong anti-BMP activity (Belo et al., 1997; Biben et al., 1998). Other secreted factors have also been shown to share this biochemical activity: chordin, noggin, and follistatin (Iemura et al., 1995; Piccolo et al., 1996; Zimmerman et al., 1996; Piccolo et al., 1999). This antagonism generates a graded inhibition of ventral BMP signaling which is essential for dorsoventral patterning and neural induction in the vertebrate embryo (De Robertis and Sasai, 1996). Considering these observations we hypothesized that some other genes might be compensating for the lack of function of cerl-1 in the KO mouse. This phenomenon has been observed with the BMP inhibitors Chordin and Noggin, as described previously (Bachiller et al., 2000). Both genes are expressed in the node of the mouse embryo, at late gastrula stage. Later they are co-expressed at the level of the notochordal and prechordal plates. Remarkably, double homozygous chordin;noggin mutants present synergistic defects at the level of the forebrain development (Bachiller et al., 2000). These defects were not induced by the single mutations alone (McMahon et al., 1998; Brunet et al., 1998; Bachiller et al., 2003), meaning that some compensation was occurring when only one BMP inhibitor was missing.

This evidence led us to test whether noggin or goosecoid (McMahon et al., 1998; Brunet et al., 1998; Yamada et al., 1995) could compensate for the lack of function of cerl-1 in the mouse. Both are involved in BMP signaling inhibition and are coexpressed with cerl-1 at the level of the prechordal plate mesendoderm, and the latter also in the AVE (Belo et al., 1998). In Xenopus experiments, Xgsc represses the expression of BMP-4 in the marginal zone (Fainsod et al., 1994) and can induce the expression of chordin (Sasai et al., 1994). However, generated double mutants for both cerl-1; noggin and cerl-1; gsc did not display any synergistic phenotype (Borges et al., 2001, 2002), indicating that neither noggin nor goosecoid compensate for cerl-1 loss-of-function and that these genes do not interact genetically. These results could be indicative that the true biological relevance of cerl-1 in the mouse might not be its anti-BMP activity.

As referred before, mouse cerl-1 has a strong anti-Nodal activity (Belo et al., 2000). Studies using the chick homologue Caronte (or chick Cerberus, cCer), demonstrated that its expression in the hypoblast (the chick equivalent of the AVE in the mouse and of the ADE in Xenopus) is necessary for head formation (Bertocchini and Stern, 2002), by preventing the formation of trunk mesoderm in the prospective head neuroectoderm via its anti-Nodal activity. Lefty-1 is another nodal secreted antagonist and is expressed in AVE cells (Meno et al., 1997). Like cerl-1 KO embryos, generated mouse mutants lacking lefty-1 also lack gastrulation phenotypes (Meno et al., 1998; Belo et al., 2000). However, when cerl-1; lefty-1 double mutant animals were generated, development of the resulting embryos was greatly impaired due to excessive and unregulated nodal activity (Perea-Gomez et al., 2002; Yamamoto et al., 2004). Yamamoto and colleagues showed that asymmetric nodal inhibition directs Distal Visceral Endoderm (DVE) migration to an anterior position. This migration is accomplished by simulating the proliferation of visceral endodermal cells by Nodal while Lefty-1 and Cerl-1 determine the direction of migration by asymmetrically inhibiting Nodal activity on the prospective anterior side (Yamamoto et al., 2004). Later, this concerted inhibition of Nodal activity in the AVE is also required in order to restrict primitive streak formation to the posterior end of mouse embryos by antagonizing Nodal signaling (Perea-Gomez et al., 2002). Collectively, these studies clearly show the requirement of the nodal inhibitors cerl-1 and lefty-1, whose redundant activities during early development are essential for A-P axis development.

In conclusion, the results from Xcер, Cerl-1 and cCer studies strongly support their role in generating asymmetries at perigastrulation stages. In this context, Cerberus molecules emerge as major players in the establishment of the Anterior-Posterior axis of the early vertebrate embryo.

**Cerberus in left-right patterning**

In vertebrates, the correct development of the organs along a left-right (L-R) plane of organization is essential for the normal...
physiology of the living organism. Therefore, the formation of the left-right axis of body symmetry during embryogenesis is of major importance. The establishment of this asymmetry was shown to require the asymmetric activation of the Nodal signaling cascade in the left side of the body wall (for review see Hamada et al., 2002), the Left-Lateral Plate Mesoderm (L-LPM). During the course of vertebrate evolution, this basic feature was preserved, although some species specificities have diverged.

In the chick embryo, the first signal of morphological asymmetry is the tilt of Hensen’s node by the end of gastrulation (Dathe et al., 2002). The signaling molecule Sonic hedgehog (Shh) is expressed symmetrically within the ectoderm of Hensen’s node before HH4 (Hamburger and Hamilton 1951), the time at which it becomes restricted to the left side of the node. This is followed at HH7 by the expression of Nodal on the left side. Nodal is first expressed in a small domain of cells directly adjacent to the ones expressing Shh, and then in a large domain in the lateral plate mesoderm. Although Shh expression in the node is necessary and sufficient to induce Nodal in the non-adjacent L-LPM (Pagán-Westphal et al., 1998), the exact mechanism is largely unknown. This led to the hypothesis that there was an unknown molecule in the paraxial mesoderm (the intermediate embryonic tissue) that would transduce this information from the node towards the L-LPM.

The chick homologue of XcCer has been isolated and studied by a number of groups (cCer; Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Besides its expression in the hypoblast and anterior endomesoderm, cCer is also expressed in the left paraxial and L-LPM. Experiments using misexpression, protein-soaked beads and in vitro binding studies revealed that cCer was necessary and sufficient to transmit the Shh signal from the node to the L-LPM, leading to Nodal expression and subsequent activation of Left-Right specific gene expression (Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). In light of these properties, this novel Cerberus-like gene was denominated Caronte (Car), after the boatman in Greek mythology who ferried the souls of the dead across the River Styx. Caronte has been therefore, reported to be a second signal induced by Shh and expressed by Fgf8, and Caronte misexpression experiments suggested that it was sufficient to activate Nodal in the LPM. Caronte has been proposed to act as a BMP antagonist (Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999) activating Nodal expression in the left lateral plate mesoderm by relieving a repressive effect of BMPs on Nodal transcription.

However, Nodal molecules have been shown to induce the expression of XcCer (Osada et al., 2000) and mouse Cerl-1 (Waldrip et al., 1998; Brennan et al., 2001), which is the opposite of what has been reported for Caronte. In addition, Nodal expression on the left side of the chicken node can be observed prior to the onset of Caronte (Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Moreover, in the mouse embryo, Nodal expression in the L-LPM requires Nodal protein produced in the node (Saijoh et al., 2003; Yamamoto et al., 2003). All of these data combined leaves the relationship between Caronte and Nodal unclear, raising the possibility that the induction of Caronte expression by Shh may be mediated by Nodal and not the other way around. Moreover, Caronte has been shown to bind to Nodal (Rodriguez-Esteban et al., 1999), as the other Cerberus family members do; so if its biological activity is also similar to its ‘siblings’ it should behave as an inhibitor of nodal signaling, not an activator.

We have re-investigated the role of Caronte, hereafter denominated chicken Cerberus (cCer), in the establishment of asymmetric nodal signaling. We have implemented novel approaches in our study, namely electroporation of cDNAs, knock-down experiments using morpholino oligonucleotides against cCer, implantation of Nodal protein-soaked beads and transcriptional analysis of the cis-regulatory regions of the cCer gene. In our experiments, cCer misexpression in the node region or on the right-LPM was never able to induce Nodal, whereas cCer overexpression on the left side actually repressed Nodal. Conversely, in cCer knock-down embryos, Nodal is ectopically expressed on the right side, demonstrating that cCer acts as negative regulator of Nodal signaling (Tavares et al., 2007). We could also observe that in fact Nodal is necessary and sufficient for the induction of the transcriptional activation of the cCer gene (Tavares et al., 2007) even when targeted to the Right-LPM. We could indeed determine that the Nodal nuclear effectors FoxH1 and SMAD elements in cCer left-side enhancer are sufficient to induce the asymmetric expression on the left side of the chick embryo. Similar enhancer elements are also seen in the promoters of other asymmetically expressed Nodal responsive genes such as Leftys, Ptx2, and Nodal itself, and have been reported to be responsible for the asymmetric expression of these genes. As in the case of the mouse node (Saijoh et al., 2003), Nodal signaling in the Hensen’s node is able to induce Nodal expression in the chick L-LPM (Tavares et al., 2007). However, the induction of cCer expression by Nodal protein is faster then the induction of the Nodal one. Therefore, Nodal protein released by the node induces first cCer in a more medial domain (left paraxial mesoderm) and later Nodal in a more lateral domain (left lateral plate mesoderm).

Some of this data is reminiscent of the interactions postulated in reaction-diffusion models (Turing, 1990; Meinhardt and Gierer, 2000). Some reaction-diffusion models propose the following four interactions between an activator and an inhibitor: the activator activates its own production; the activator activates an inhibitor; the inhibitor blocks the auto-activation of the activator; the inhibitor acts long-range to restrict the effective range of the activator. The relationship between Nodal and its antagonists Lefty1 and 2 in the mouse embryo has recently been proposed to be a reaction-diffusion type, a self-enhancement and lateral-inhibition type (Nakamura et al., 2006). cCer may therefore share some of the regulatory properties of Leftys that have been described recently.

In conclusion, this data strongly supported the view that Nodal is the intermediary signal that transfers the asymmetric information from the node to the lateral plate (Pagán-Westphal and Tabin, 1998) and that the role of cCer is to confine Nodal signaling to the left side through a negative feedback mechanism, preventing Nodal signals from crossing to the right side of the chicken embryo (Tavares et al., 2007). Furthermore, given the similarities between the expression patterns and functions of chick Cer and mouse Lefty2 (for a review, see Juan and Hamada, 2001), we proposed that in the chick, cCer has taken the role of mouse’s Lefty2 in left-right patterning, and acts in addition to the midline barrier to confine Nodal signaling to the left side.

Recently, novel cerberus molecules have been isolated in mouse (cerberus-like2, Cerl-2) and zebrafish (charon, Marques et al., 2001).
et al., 2004; Hashimoto et al., 2004). Cerl-2 is closely related to mouse cerberus-like (I=35%, P=47%), cCerberus (I=34%, P=51%), Xcoco (I=38%, P=53%), zebrafish charon (I=33%, P=55%) and to human hCer-2 (I=57%, P=65%). Functional analysis revealed that both genes play an important role in the establishment of Left-Right asymmetry (Marques et al., 2004; Hashimoto et al., 2004). Cerl-2 displays a unique asymmetric expression domain around the mouse node, being asymmetrically expressed on the right side. cerl-2 transcripts can be firstly detected in a horse-shoe shaped pattern in the perinodal region of the E7.0 mouse embryo, resembling Nodal expression at this stage (Lowe et al., 1996; Collignon et al., 1996). However, by late headfold stage, expression of cerl-2 begins to decrease in intensity on the left side and by E8.0 is mostly detected in the right side of the node assuming a complementary expression pattern to the one of Nodal at this stage (Lowe et al., 1996; Collignon et al., 1996). We demonstrated that Cerl-2 is a potent antagonist of Nodal signaling by directly binding to Nodal protein (Marques et al., 2004). Analysis of a generated mutant mouse line in which we deleted the Cerl-2 gene revealed that 1/3 of the cerl-2−/− newborns died within the first 48 hours after birth and displayed left pulmonary isomerism, thoracic abdominal organs (Marques et al., 2004). When analyzed during early somitogenesis, we detected that the Cerl-2 loss of function leads to bilateral expression of the left side-specific genes Nodal, Lefty-2 and Pitx2 (Marques et al., 2004).

In light of this data, the role of Cerl-2 is to restrict nodal activity to the left side of the node, preventing additional activation of Nodal, Lefty-2 and Pitx-2 in the R-LPM. In the absence of Cerl-2 antagonistic activity on the node, Nodal can now also be activated in the R-LPM, leading to bilateral expression of this genetic cascade. These observations highlight the function of cerl-2 in the tight regulation of Nodal activity in the node, indicating that Cerl-2 plays an important role in the early symmetry breaking events that take place in the node.

In mice, a model has been proposed in which monocilia protruding from cells in the late gastrula node generate a left-right flow of extracellular fluid that results in the establishment of asymmetric gene expression (Nonaka et al., 1998). This “nodal flow” is disturbed in several mouse mutant lines that display L-R phenotypes, which support its role in the establishment of correct left-right body axis (Nonaka et al., 1998; Marszalek et al., 1999). The work on Cerl-2 suggests that, in the mouse, L-R asymmetry is controlled by a double-assurance mechanism consisting of two systems working in parallel, the first relying on the leftward nodal-cilia flow, and the second, on the antagonism between Cerl-2 and Nodal.

In the zebrafish embryo, Kupffer’s vesicle has been demonstrated to be the functional equivalent of the mouse node (Essner et al., 2002, 2005), with motile cilia that create a directional flow fluid just prior to the onset of asymmetric gene expression in lateral cells. Disruption of this flow impairs correct L-R patterning (Essner et al., 2005). Zebrafish Charon is expressed in the Kupffer’s vesicle at the 10-somite stage (14 hpf; Hashimoto et al., 2004). Charon expression pattern assumes a horse shoe-shape, resembling the one from Cerl-2 at initial stages (Marques et al., 2004). But in zebrafish, expression of southpaw (the equivalent of mouse Nodal) and Charon are not asymmetric in the vicinity of the Kupffer’s vesicle. Functional assays using microinjection of Charon mRNA in zebrafish embryos demonstrated that the dorsalizing activity of all of the three known zebrafish Nodal-related molecules (southpaw, Cyclops and squint; Sampath et al., 1998; Long et al., 2003; Rebagliati et al., 1998) can be inhibited by Charon. Similarly to southpaw, expression pattern of the cerl-2mutants, down-regulation of Charon by morpholino oligonucleotides lead to disturbs in the correct establishment of the Left-Right axis. Those included bilateral expression in the LPM of the left side-specific genes southpaw, cyclops, lefty2 and pitx2 (Hinshimoto et al., 2004), and defects in asymmetric heart development.

Recently it has been reported that Xenopus Coco also plays an important role in the establishment of the L-R axis of the Xenopus embryo (Vonica et al., 2007). Coco has been found to be a nodal antagonist, that is expressed bilaterally in the posterior paraxial mesoderm at neurula stage, where it shares the same expression pattern with Xnr1 and derrière. Experiments using Coco morpholinos demonstrated that Coco is required exclusively on the right side and Xnr1 on the left side, for proper Left-Right patterning (Vonica et al., 2007). Taken together, these results suggest that Charon and Coco might be true orthologues of cerl-2. And as in the case of the mouse embryo, the antagonistic activity of the Cerberus molecules Charon and Coco, against Southpaw and Xnr1 (Nodal molecules), plays an important role in the establishment of the L-R axis in zebrafish and Xenopus embryos.

Although chick Cer, Xenopus Coco, zebrafish Charon and mouse Cerl-2 have different expression patterns, the Cerberus-like proteins encoded by these genes seem to have an evolutionary conserved mechanism of Nodal antagonism in the vertebrate embryo. This conserved mechanism, crucial for correct L-R development, is to restrict Nodal signaling in the left

Fig. 1. Cerberus prevents Nodal signaling in the anterior and right sides of the vertebrate embryo in order to promote head formation and asymmetric distribution of the internal organs.
side of the vertebrate embryo.

Final remarks

10 years after the isolation of Xcerberus (Bouwmeester et al., 1996), the founding member of this still increasing family, Cerberus-like molecules emerge as key regulators of Nodal signaling. Although not signaling proteins per se, they are very important regulatory molecules, playing a primordial role in restricting Nodal signaling both in space and time. This data suggests that one efficient way of creating asymmetries in the early developing embryo is by restricting the activation of certain signals in a specific region in time. It is becoming more and more evident that biological processes like the generation of asymmetries arise from complex regulatory mechanisms, and inhibitory molecules like the Cerberus-family members are not secondary players in these pathways, but can now claim their intrinsic importance as generators of developmental processes. Our observations confirm the increasing body of evidence that patterning of the vertebrate embryo results mostly from antagonistic protein-protein interactions in the extracellular space (Fig. 1), being the Cerberus-like family of secreted inhibitors a crucial player in this process.

Bearing this in mind, and in order to further characterize the molecular mechanisms that play a role in the early A/P axis establishment, we have been using Cerberus-family members as a ‘bait’ to uncover potential new players. We have performed a differential screening using a generated transgenic mouse line in which EGFP is expressed in the AVE, under the control of the promoter region of the Cerf-1 gene. Gene expression profiling using GeneChips® (Affymetrix®) identified novel differentially expressed transcripts at the very early stages of A-P axis establishment (Mário Filipe, unpublished), some of them now currently being studied at our laboratory (Silva et al., 2006; Filipe et al., 2006; Salgueiro et al., 2006). We expect that this approach will allow us to learn more about asymmetry generation, tissue patterning and specification during early vertebrate development and that this knowledge can be applied in the future to regenera-tive medicine.

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