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

JOSÉ ANTÓNIO BELO[1,2,3,*], ANA C. SILVA[1,2], ANA-CRISTINA BORGES[1,2], MÁRIO FILIPE[1], MARGARET BENTO[1,2], LISA GONÇALVES[1,2], MARTA VITORINO[2], ANA-MARISA SALGUEIRO[1,2], VERA TEXEIRA[1], ANA T. TAVARES[1] and SARA MARQUES[1,2]

[1]Instituto Gulbenkian de Ciência, Oeiras, Portugal and [2]IBB-Institute for Biotechnology and Bioengineering, Centro de Biomedicina Molecular e Estrutural, Universidade do Algarve, Campus de Gambelas, Faro, Portugal

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 uncommited 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 Anterio-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 transplantation of the dorsal blastopore lip of a newt embryo into the ventral (opposite) side of a host embryo, generated the formation of a new body axis, or Siamese twin (Spemann and Mangold, 1924). Furthermore, they observed that this 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-

***Address correspondence to:** José António Belo.CBME/FERN, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. Fax +351-289-818-419. e-mail: jbelo@ualg.pt

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tion. The organizer cells express several transcription factors, like goosecoid, Siamois, HNF3β, Xtwn, Xlim-1, Xnot, and secreted factors with "dorsalizing activity", like Chordin, Noggin, Follistatin, Frzb-1, crescent, SFRPs and DKK-1. Several of the secreted molecules by the organizer to the extracellular space bind to and inhibit the activity of "ventralizing factors" namely BMP's and Wnt8, giving identity to the ectodermal and the mesodermal cells (see De Robertis *et al.*, 1997; De Robertis and Kuroda, 2004). Among the newly identified antagonizers, one excelled due to its remarkable properties: *Xenopus* cerberus (*X*cer). This novel secreted protein is expressed in the most anterior tip of the non-involuting yolky 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 Cerberus (*cCer*; 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 c*Cer* 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 c*Cer* 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 c*Cer* 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 (*endodermin*) 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 *XCerberus* mRNA to induce the referred endodermal and heart markers. *XCerberus* 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 DiI (Bouwmeester *et al.*, 1996).

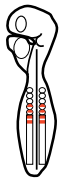
In an effort to determine the genetic and biochemical basis of the head induction by XCer, Piccolo and colleagues demonstrated that Cerberus functions as a multivalent growth-factor

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 signals (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

EXPRESSION PATTERN AND FUNCTION OF THE CERBERUS FAMILY MEMBERS

Gene	Peri-Gastrulation	Post-Gastrulation	Function	References
xcer			Nodal, Bmp and Wnt antagonist. Head induction and specification. Inhibition of mesoderm signals in the ADE.	Bouwmeester *et al.*, 1996 Piccolo *et al.*, 1999 Silva *et al.*, 2003 Kuroda *et al.*, 2004
mcer-l			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.	Belo *et al.*, 1997 Belo *et al.*, 2000 Perea-Gomez *et al.*, 2002 Yamamoto *et al.*, 2004
ccer			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.	Rodriguez-Esteban *et al.*, 1999 Bertocchini and Stern, 2002 Tavares *et al.*, 2007
xcoco			Bmp, Nodal, Activin, Derriére and Wnt antagonist. Regulates cell fate specification and competence prior to onset of neural induction. Required to restrict TGF-β signaling to the left side of the embryo.	Bell *et al.*, 2003 Vonica and Brivanlou, 2007
zcharon			Nodal antagonist. Required to restrict Nodal signaling to the left side of the embryo.	Hashimoto *et al.*, 2004
mcerl-2			Nodal and Bmp antagonist. Required in the node to restrict Nodal signaling activation to the left lateral plate mesoderm.	Marques *et al.*, 2004

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*, c*Cer*), 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;lefty1* 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 *Lefty1* 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 *lefty1,* whose redundant activities during early development are essential for A-P axis development.

In conclusion, the results from *Xcer, Cer-l* 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 *XCer* 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 secondary signal induced by Shh and repressed 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 *XCer* (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 asymmetrically expressed Nodal responsive genes such as Leftys, Pitx2, 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., 2004; Hashimoto *et al.*, 2004). Cerl-2 is closely related to mouse cerberus-like (I=35%, P=47%), cCerberus (I=34%, P=51%), *X*coco (I=38%, P=53%), zebrafish charon (I=33%, P=55%) and to human *h*Cer-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 *situs inversus* and cardiovascular malformations, being the latter their probable cause of death. The mutant animals that survived become normal adults although 1/4 die between weaning age and 3 months old, most of them showing heterotaxia of the 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*, *Lefty2* 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.

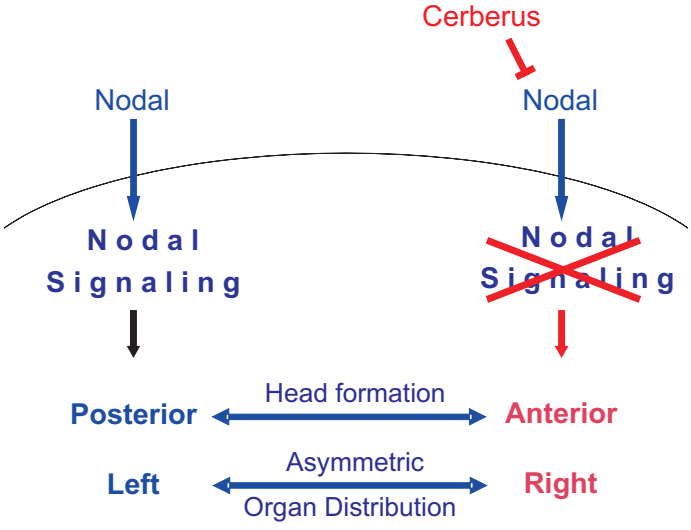


Fig. 1. Cerberus prevents Nodal signaling in the anterior and right sides of the vertebrate embryo *in order to promote head formation and asymmetric distribution of the internal organs.*

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 fluid flow 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 the phenotype of the *cerl-2* mutants, 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* (Hashimoto *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 *derriére*. 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 body 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

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 *Cerl*-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 regenerative medicine.

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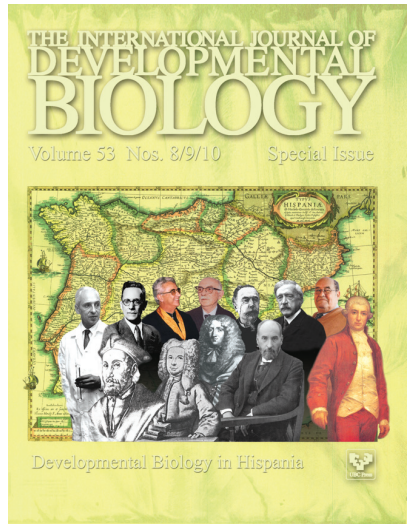

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