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Award Number: DAMD17-96-1-6323

TITLE: Role of the EGF-Related Growth Factor Cripto in Murine Mammary Tumorigenesis

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REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Role of the EGF-Related Growth Factor Cripto in Murine Mammary Tumorigenesis

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We have been investigating the biological and biochemical functions of the Cripto gene, which encodes an extracellular protein that is a member of the EGF-CFC gene family, and which has been implicated in autocrine or paracrine signaling during human breast carcinogenesis. To elucidate the potential role of Cripto in mammary development and tumorigenesis, we have been investigating the in vivo activities of Cripto using transgenic mice that overexpress Cripto in the mammary gland, and have been investigating the molecular mechanisms of CRIPTO protein signaling. In the past year, we have generated molecular genetic and biochemical lines of evidence that Cripto acts as an essential co-factor for signaling by the divergent TGF-β factor Nodal. These findings provide a model for potential mechanisms of Cripto function through modulation of specific TGF-β-related signals in mammary development and tumorigenesis.
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5. Introduction:

Our work on breast development and tumorigenesis has focused on the *Cripto* gene, which encodes an extracellular protein that is a member of the EGF-CFC gene family. Previous studies have suggested that *Cripto* may be involved in autocrine or paracrine signaling during human breast carcinogenesis (reviewed in [1]). Thus, *Cripto* is consistently overexpressed in a large percentage of human breast cancers, and is not expressed at high levels in normal breast tissue [2], and *Cripto* has transforming activity when overexpressed in NOG-8 mouse mammary epithelial cells [3]. To elucidate the potential role of *Cripto* in breast development and tumorigenesis, we have been investigating the activities of *Cripto* in mammary development using transgenic mice and cell culture systems, and have been investigating the molecular mechanisms of CRIPTO protein action, with the objective of identifying a putative CRIPTO receptor(s).

6. Body:

In the past year, we have made significant progress towards understanding the molecular mechanisms of *Cripto* function, which has led to an important revision of our initial view of *Cripto* encoding an EGF (epidermal growth factor)-related growth factor. Based on recent molecular genetic data from our lab and others, it now appears that *Cripto* acts as an essential co-factor for signaling by a divergent member of the TGF-β (transforming growth factor-beta) superfamily.

6.a. Review of recent work relevant for our studies

To understand the biological and biochemical basis for *Cripto* function, we have been investigating its role in normal development. We initially identified the EGF-CFC family through studies of mesoderm formation during mouse gastrulation, in which we isolated a novel gene that we named Cryptic ([4]; see appendix), based on its similarity to murine and human *Cripto* [5, 6]. Mammalian *Cryptic* and *Cripto*, frog *FRL-1*, and zebrafish *oep* encode extracellular proteins [4, 7] that share an N-terminal signal sequence, a variant EGF-like motif, a novel conserved cysteine-rich domain (that we named the CFC ([*Cripto*, *Frl-1*, and *Cryptic*) motif]), and a C-terminal hydrophobic region (Fig. 1). Although the overall level of sequence conservation is relatively low (approximately 30% identity), all EGF-CFC family members appear to have functionally similar activities in assays for phenotypic rescue of *oep* mutant fish embryos by mRNA microinjection [8].

![Sequence alignment of EGF-CFC family members. Amino acid residues conserved among all family members are boxed, while residues conserved among four out of five members are shaded. The positions of the variant EGF-motif and the novel CFC motif are indicated. Locations of the predicted sites for N-terminal signal sequence cleavage are indicated by vertical bars; asterisks denote the positions of the N-terminal epitope tag used in expression constructs for mouse *Cripto* and *Cryptic*. Shaded bars indicate the positions of C-terminal truncations constructed for EGF-CFC proteins.](image-url)
In our work, we have gained essential insights into the biological functions and potential biochemical activities of EGF-CFC proteins through analysis of knock-out mice for 

**Cripto** and **Cryptic**. We have found that the roles of **EGF-CFC** genes in axis formation are neatly divided in the mouse embryo, so that **Cripto** is required for correct orientation of the A-P (anterior-posterior) axis, while **Cryptic** is necessary for determination of the L-R (left-right) axis ([9, 10]; see appendix). These recent studies have led to a revised model for the biochemical activities of EGF-CFC proteins.

Thus, in contrast to previous models of CRIPTO protein having growth factor activity (reviewed in [1]), recent lines of evidence from molecular genetic studies indicate that EGF-CFC proteins act as essential co-factors for a signaling factor known as NODAL. The **Nodal** gene encodes a signaling factor that is a divergent member of the TGF-β superfamily, and displays a mutant phenotype similar to that for **Cripto** [11, 12]. The downstream signaling pathway for **Nodal** has primarily been suggested by our current understanding of TGF-β signal transduction pathways (reviewed in [13]), and by gene targeting experiments in mice that have demonstrated similar and/or synergistic phenotypes for targeted disruption of **Nodal**, the type II activin receptor **ActRIIB**, the type I receptor **ActRI**B, and the cytoplasmic signal transducer **Smad2** [12, 14-18]; however, there is no biochemical evidence at present that NODAL protein directly binds to and activates activin receptors. Recent work has led to the proposal that NODAL and EGF-CFC proteins are inactive by themselves, while in combination their activity is similar to that of activin [8]. Moreover, the phenotypes of both **Cripto** and **Cryptic** mutant mice can be readily interpreted as resulting from defects in **Nodal** signaling [9, 10]. These data can be summarized in terms of a possible regulatory pathway for **Nodal** and **EGF-CFC** activities (Fig. 2).

These apparently discrepant findings can be resolved by a simple model in which EGF-CFC proteins form membrane-associated components of a receptor complex, and mediate **Nodal** signaling through signal-transducing partner(s) that may include activin receptors [8]. However, in principle, release of EGF-CFC proteins from the membrane might result in a freely diffusible protein, which could form part of a receptor complex on a neighboring cell that may not itself express the **EGF-CFC** gene, in effect acting as a signal. Such a mechanism has been shown to be the case with the GFRα protein, which is tethered to the membrane via a glycosyl-phosphatidylinositol (GPI) linkage and heterodimerizes with the c-RET tyrosine kinase receptor to form a receptor for GDNF, another distant member of the TGF-β superfamily [19, 20]. In this view, the downstream signaling effects documented for CRIPTO protein might represent cross-talk between EGF receptor and SMAD2 signaling pathways, which has been previously documented [21, 22].

### 6.b. Technical Objective I: Generation and analysis of **Cripto** transgenic mice

**Rationale:** To investigate the effects of **Cripto** on mammary development and oncogenesis, we have generated transgenic mice that overexpress **Cripto** in the mammary epithelium. Our previous studies had shown that **Cripto** is expressed at extremely low levels throughout mammary development, with slightly elevated expression during pregnancy and lactation. To overexpress **Cripto**, we have produced a modified **Cripto** transgene that should direct high-level secretion. We have previously shown that EGF-CFC proteins, including CRIPTO, are poorly secreted from
transfected cells in culture, probably because the endogenous signal sequence is non-conventional and directs inefficient secretion ([4]; data not shown). Moreover, CRIPTO protein appears to be membrane-associated due to the presence of the C-terminal hydrophobic region (see below). Since the biological activity of a Cripto transgene should depend upon the levels of protein secretion attained in vivo, we have constructed a transgene expression vector containing a heterologous efficient signal sequence and encoding a C-terminally truncated protein.

Results: To direct expression of CRIPTO transgenes to the mammary epithelium of transgenic mice, we utilized the MMTV-SVPA vector [23], which contains a mouse mammary tumor virus (MMTV) long terminal repeat (LTR) enhancer/promoter (Fig. 3a). We have subcloned a modified mouse Cripto cDNA (sec-Cripto) into this vector (Fig. 3b). We have successfully generated five founder mice carrying this MMTV-sec-Cripto transgene, and have established stable lines from each of these founders.

![Diagram of transgene vector and inserts](attachment:image.png)

**Figure 3.** Schematic depiction of the transgene vector and inserts. *a,* The MMTV-SVPA expression vector contains the long terminal repeat from the mouse mammary tumor virus, 5' untranslated sequences from ras, and the 3' polyadenylation region from SV40 [23]. *b,* The murine Cripto transgene inserts have been produced in unmodified and modified (Sec-Cripto) forms. For the modified construct, we have utilized the leader peptide from a murine immunoglobulin kappa chain gene (from the pSecTagB expression vector; Invitrogen) as a replacement for the endogenous CRIPTO leader peptide.

To analyze these transgenic lines, we have examined transgene expression and phenotype at different stages of mammary development, thereby accomplishing tasks 1-5 of our Statement of Work. Four of the five MMTV-sec-Cripto transgenic lines display transgene expression in the mammary gland in virgin female animals as well as during pregnancy and lactation (data not shown). However, examination of whole-mounts of mammary fat pads from adult transgenic females revealed no phenotypic abnormalities in virgin, pregnant, lactating, or involuting animals (data not shown). At present, the absence of any detectable phenotype suggests that Cripto expression alone is not sufficient to alter mammary epithelial differentiation. We are continuing to monitor these MMTV-sec-Cripto mice to determine whether they will develop a mammary phenotype with advancing age or multi-parous status.

These results are consistent with our model that EGF-CFC proteins act as essential co-factors for Nodal signaling, and with the finding that broad ectopic overexpression of oep in fish did not result in embryonic phenotypes [7]. Our current interpretation is that the ectopic expression of Cripto in human breast tumors may reflect a cooperative interaction with overexpressed members of the TGF-β family, perhaps including Nodal or related genes. To test this hypothesis, we are now attempting to generate MMTV-Nodal transgenic mice, and to cross these mice with the MMTV-sec-Cripto transgenics to examine the resulting phenotype.
6.c. Technical Objective II: Expression of Cripto in cell culture model systems

Rationale: To examine the role of Cripto in cellular proliferation, differentiation and transformation, we have been utilizing retroviral gene transfer as a strategy to efficiently transfect Cripto into relevant cell lines. In studies reported in the previous update, we have focused on cell lines that have been reported to respond to exogenous human CRIPTO protein, such as the HC-11 mammary epithelial cell line [24, 25], and have made significant progress towards tasks 8-10.

Based on the apparent lack of phenotype of the MMTV-sec-Cripto transgenic mice, however, we have decided not to continue our experiments on mammary cell lines until we have acquired a better understanding of the biochemical basis of CRIPTO protein function. We are currently undertaking a survey of expression of Nodal and closely related TGF-β factors (such as Vg1) in a range of mammary cell lines, with the expectation that expressing cell lines will be more likely to display phenotypic changes in response to Cripto expression. These experiments will provide the preliminary data necessary to test the hypothesis that co-expression of Cripto with specific TGF-β factors may play a critical role in mammary differentiation and/or tumorigenesis.

6.d. Technical Objective III: Biochemical analysis of CRIPTO function

Rationale: Despite the present lack of understanding as to the potential mechanism(s) by which EGF-CFC and Nodal activities might interact at the molecular level, understanding the biochemical basis of this interaction is of fundamental importance. Therefore, we have undertaken to (i) characterize the cellular localization of CRIPTO protein, (ii) produce and purify active CRIPTO protein for biochemical analyses, and (iii) examine potential interactions between CRIPTO and NODAL proteins. These experiments form the basis for elucidating the biochemical mechanism of CRIPTO function.

Results: (i) Membrane-association of CRIPTO protein: Previous studies have suggested that members of the EGF-CFC family encode extracellular proteins that are localized to the surface of transfected cells, with this association mediated by the C-terminal hydrophobic domain [4, 7]. Therefore, we have investigated whether CRIPTO is in fact a secreted protein that is membrane-associated. Our previous work had demonstrated that CRYPTIC protein underwent signal sequence cleavage and N-linked glycosylation when expressed by in vitro transcription/translation in the presence of microsomal membranes [4]. However, since the C-termini of EGF-CFC proteins are not conserved in their amino acid sequences, but only in their hydrophobicity (Fig. 1), it was unclear whether mammalian CRIPTO and would be membrane-associated via its C-terminus, as has been shown for Oep protein [7]. Moreover, in previous experiments using alkaline phosphatase-CRYPTIC fusion proteins, we had detected very low levels of fusion protein in culture supernatants of transfected COS cells [4].

To determine whether CRIPTO protein is indeed secreted and membrane-associated, we investigated its cellular localization in transfected mammalian cells using immunofluorescence detection of epitope-tagged proteins (data not shown), and obtained results similar to that described for Oep [7]. Furthermore, we have extended these findings by cellular fractionation experiments, using insect cells infected with baculoviruses expressing a secreted CRIPTO protein fused at its N-terminus to glutathione-S-transferase (GST) (Fig. 4). In this experiment, we found that purified plasma membrane fractions contained both full-length and C-terminal truncations of the GST-CRIPTO fusion protein, as detected by Western blotting using a specific anti-CRIPTO antiserum. In contrast, the culture supernatants from infected cells contained high levels of the truncated protein, but only barely detectable levels of the full-length protein. These results support the idea that full-length EGF-CFC proteins are associated with the cell membrane, while C-terminal
truncated proteins can be secreted into culture supernatants. At present, however, it remains unclear whether full-length EGF-CFC proteins remain associated with the cell surface through a C-terminal transmembrane domain, through tight association with other membrane proteins, or through a lipophilic modification.

**Figure 4.** Western blot detection of CRIPTO protein in plasma membrane fractions and culture supernatants prepared from baculovirus-infected insect cells. Arrows at right indicate positions of full-length and truncated GST-RIPTO fusion proteins. **Methods.** For expression of secreted GST-RIPTO fusion proteins, Cripto cDNAs corresponding to the mature protein with an intact or truncated C-terminus (as shown in Fig. 2) were cloned into the pAcSecG2T transfer vector (Pharmingen), which contains a gp67 signal sequence for high-level secretion, and generates an N-terminal fusion with GST. Baculovirus stocks were prepared in Sf9 insect cells and used to infect High-5 insect cells (Invitrogen). Plasma membrane fractionation was performed essentially as described [26, 27]. The absence of contaminating endoplasmic reticulum in the plasma membrane fractions was confirmed using assays for cytochrome b, reductase (not shown). Rabbit polyclonal antisera specific for CRIPTO were generated (Covance) by immunization with GST-RIPTO fusion proteins expressed in *E. coli* and purified using glutathione-agarose beads. The antisera were characterized for specificity in Western blotting experiments using EGF-CFC proteins expressed in bacterial and mammalian cells.

**Expression of active CRIPTO protein in insect cells:** To achieve expression of active CRIPTO protein, we employed a baculovirus expression strategy that would result in secretion of CRIPTO into the culture supernatant of infected insect cells. For this purpose, we utilized the pMelBac baculovirus transfer vector (Invitrogen), which contains a heterologous signal sequence, to express C-terminally truncated CRIPTO protein (without epitope tags) at high levels in culture supernatants. To enable rapid and efficient protein purification, we infected High-5 insect cells (Invitrogen) grown in defined serum-free media; by adjusting the time course and multiplicity of infection, we determined optimal conditions for harvesting culture supernatants to minimize contamination from cytoplasmic proteins released by lysed cells. We were able to achieve efficient purification of CRIPTO from culture supernatants using a Sephadex G-75 gel filtration column (Fig. 5a). As shown in Fig. 5b, c, we were able to obtain at least 90% pure CRIPTO protein in specific column fractions, as judged by examination of silver stained gels and by Western blotting using a specific CRIPTO polyclonal antisera. In subsequent experiments, we have scaled-up the purification protocol and have obtained approximately 400 μg of purified CRIPTO protein from 1 liter of insect cell culture supernatants.

**Figure 5.** Purification of C-terminal-truncated CRIPTO protein from culture media of baculovirus-infected insect cells. a, Elution profile from Sephadex G-75 gel filtration column, showing UV absorbance plotted
against fraction number. b, Silver stained gels of indicated column fractions. c, Western blot detection of CRIPTO protein in column fractions shown in (b). At right, large arrows mark position of intact C-terminal-truncated CRIPTO protein, while small arrows indicate position of a faster migrating CRIPTO product. The position of pre-stained molecular size standards at 25.4, 20, 14, and 9.3 kDa are indicated at left. Note that the purified C-terminal-truncated CRIPTO protein migrates at approximately 14 kDa relative to these size standards; the calculated molecular weight is 15 kDa, and the measured value by gel filtration is approximately 17 kDa. Methods. A CRIPTO cDNA corresponding to the mature protein with C-terminal truncation was cloned into the pMelBac transfer vector (Invitrogen), which contains a mellitin signal sequence for high-level secretion. This construct was used to prepare high-titer (2 x 10^8 pfu/ml) baculovirus stocks in Sf9 insect cells following the supplied protocol (Invitrogen). For protein purification, High-5 insect cells (Invitrogen) were grown under serum-free conditions (Ex-Cell 405 media; JRH Biosciences), and infected at an MOI of 0.4, followed by culture for 72 h at 27°C. After harvest of culture supernatants, 0.1 mM PMSF, 2 mM EDTA, and 0.8 mM DTT (final concentrations) were added, followed by immediate application to a 2.5 by 120 cm Sephadex G-75 column that had been equilibrated with 50 mM HEPES buffer, pH 6.5, 0.1 mM PMSF, 2 mM EDTA, 0.8 mM DTT, and 150 mM NaCl. Fractions (5 ml) were collected and analyzed on 15% gels by SDS-PAGE, followed by silver staining or Western blotting. The fractions containing intact C-terminal truncated CRIPTO protein (numbers 26 to 29) were concentrated to 300 μl using centrifugal filter concentrators (Millipore).

To demonstrate the activity of our purified CRIPTO protein, we have employed a straightforward assay for CRIPTO activity that has been previously described by Salomon and colleagues [24, 28]. This assay uses HC-11 mammary epithelial cells to measure activation of p42/44 MAP kinase, which undergoes phosphorylation within five minutes of addition of active CRIPTO protein to the culture media [28]. Using this assay for CRIPTO activity, we showed that addition of purified CRIPTO protein resulted in significant MAPK phosphorylation (Fig. 6). As a positive control, we showed that addition of EGF was also effective (Fig 6).

**Figure 6.** Activity of CRIPTO protein in the MAPK activation assay. Shown is Western blot detection of phosphorylated p42/44 MAPK protein in cell lysates of untreated HC-11 cells (− control), or of HC-11 cells that were treated with EGF (+ control), or with purified CRIPTO protein. Arrows at right indicate positions of phosphorylated MAPK. The position of molecular size standards at 61.5, 50.8, and 37.6 kDa are indicated by dashes at left. Methods. The MAPK activation assay was performed as described [24, 28]. We detected p42/44 MAPK phosphorylation in cell lysates of HC-11 cells using a monoclonal antibody that is specific for the phosphorylated protein (New England Biolabs); 150 ng/ml epidermal growth factor (Sigma) was used as a positive control for activation.

(iii) Evidence for an interaction between CRIPTO and NODAL proteins: As a first step for investigating NODAL protein function, we have made constructs that express secreted processed NODAL protein in transfected mammalian cells. For this purpose, we have used a wild-type epitope-tagged Nodal construct containing an unmodified prodomain, as well as a chimeric protein with a heterologous BMP-4 prodomain. We have found that processing of unmodified NODAL protein is dependent upon the cell line used for expression, which presumably reflects differential expression of the specific pro-protein convertase(s) required for NODAL processing [29]. Thus, while NODAL protein is not processed successfully in transfected COS cells (data not shown), a cleaved product of the expected size can be found in the culture supernatant of transfected 293T cells (Fig. 7). We are presently seeking to determine whether our secreted NODAL protein displays activity as a dorsalizing factor in frog animal cap assays [30, 31].
Figure 7. Production of cleaved NODAL protein in mammalian cells. Expression constructs that encode a chimeric BMP4 prodomain fused to the NODAL mature region, or a full-length unmodified NODAL, were FLAG epitope-tagged at their C-terminus. Following transient transfection of 293T cells, culture supernatants were concentrated 10-fold and used for Western blotting with a FLAG antiserum (Sigma). Positions of size standards at 50.3, 37.6, 25.4, 20, 14, and 9.3 kDa are indicated.

Using our purified CRIPTO protein as well as epitope-tagged CRIPTO and NODAL proteins produced in culture supernatants of transfected 293T cells, we have obtained preliminary evidence from co-immunoprecipitation experiments for a direct binding interaction between CRIPTO and NODAL. In the example shown in Fig. 8, we have mixed HA-tagged CRIPTO protein and FLAG-tagged NODAL protein (produced either as a BMP-prodomain chimera or with its native prodomain) in solution, and immunoprecipitated with anti-HA antibody-coupled beads. A specific band corresponding to the mature NODAL product is detected by Western blotting using an anti-FLAG antibody only when both CRIPTO and NODAL proteins are present (Fig. 8). Similar results have been obtained using HA-tagged NODAL and FLAG-tagged CRIPTO proteins, as well as with HA-tagged NODAL and untagged CRIPTO purified from insect cells detected by an anti-CRIPTO antiserum (data not shown). We are currently verifying and extending these results by investigating the interaction of NODAL protein with other members of the EGF-CFC family, and with various mutant forms of CRIPTO. We are also investigating whether a NODAL/CRIPTO protein complex is able to bind to a soluble extracellular domain of ActRIIB, and are attempting to establish a cell culture assay for NODAL activity, using published assays for activin activity.

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Figure 8. Co-immunoprecipitation of CRIPTO and NODAL proteins. Epitope-tagged CRIPTO-HA and NODAL-FLAG proteins were produced in culture supernatants of transiently transfected 293T cells, followed by 8-fold concentration. Protein complexes were immunoprecipitated by incubation with anti-HA monoclonal antibody-coupled beads (Covance), and washed 4 times with 1x PBS/0.1% NP-40. Immunoprecipitated complexes were resolved on a 12% SDS-PAGE gel and Western blotted using an anti-FLAG monoclonal antibody (Sigma). Positions of size standards at 81.6, 61.5, 50.8, 37.6, 25.4, 20, 14, and 9.3 kDa are indicated.

Our preliminary results suggest that CRIPTO acts as a co-factor for NODAL signaling by a direct protein binding interaction, which presumably mediates signaling through activin receptors. These results imply that CRIPTO may not have a cell-surface receptor, but instead functions solely through interaction with members of the TGF-β family. Thus, we have essentially fulfilled Tasks 13-18 of our Statement of Work.
7. Key Research Accomplishments:

- Analysis of Cripto and Cryptic mutant phenotypes supports an interaction of EGF-CFC genes with the Nodal signaling pathway.
- Transgenic mice overexpressing Cripto in the mammary gland have not displayed an overt morphological phenotype to date, consistent with the lack of activity of EGF-CFC genes in isolation, and their function as co-factors for Nodal.
- Production of purified CRIPTO protein and mature NODAL protein has led to the preliminary identification of a potential binding interaction between these proteins.

8. Reportable Outcomes:

See attached manuscript in appendix (Yan et al. (1999). Genes Dev. 13, 2527-2537).

9. Conclusions:

In the past year, we have made important progress in investigating the molecular basis of Cripto function. We have generated several lines of evidence that Cripto acts as an essential co-factor for Nodal signaling, and conversely, that Cripto activity requires interaction with Nodal and possibly other divergent members of the TGF-β family. These results are supported by our preliminary data indicating a direct binding interaction between CRIPTO and NODAL proteins. Taken together, our results have provoked a substantial revision of our views of Cripto function, and provide fresh insights into potential mechanisms of Cripto activity in mammary development and tumorigenesis.

10. References:


Conserved requirement for *EGF-CFC* genes in vertebrate left-right axis formation

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Specification of the left-right (L-R) axis in the vertebrate embryo requires transfer of positional information from the node to the periphery, resulting in asymmetric gene expression in the lateral plate mesoderm. We show that this activation of L-R lateral asymmetry requires the evolutionarily conserved activity of members of the *EGF-CFC* family of extracellular factors. Targeted disruption of murine *Cryptic* results in L-R laterality defects including randomization of abdominal situs, hyposplenia, and pulmonary right isomerism, as well as randomized embryo turning and cardiac looping. Similarly, zebrafish *one-eyed pinhead (oep)* mutants that have been rescued partially by mRNA injection display heterotaxia, including randomization of heart looping and pancreas location. In both *Cryptic* and *oep* mutant embryos, L-R asymmetric expression of *Nodal/cyclops, Lefty2/antivin, and Pitx2* does not occur in the lateral plate mesoderm, while in *Cryptic* mutants *Lefty1* expression is absent from the prospective floor plate. Notably, L-R asymmetric expression of *Nodal* at the lateral edges of the node is still observed in *Cryptic* mutants, indicating that L-R specification has occurred in the node but not the lateral plate. Combined with the previous finding that *oep* is required for *nodal* signaling in zebrafish, we propose that a signaling pathway mediated by *Nodal* and *EGF-CFC* activities is essential for transfer of L-R positional information from the node.

[Key Words: Left–right asymmetry; isomerism; heterotaxia; node; lateral plate mesoderm; *Nodal*]

Received July 14, 1999; revised version accepted August 13, 1999.

Of the three major body axes, the left-right (L-R) axis is the last to be determined during vertebrate embryogenesis. The initial specification of the L-R axis is likely to begin during late stages of gastrulation, but tissue-specific manifestations of morphological L-R asymmetry become apparent much later in development, throughout organogenesis into the late fetal period (for review, see Ramsdell and Yost 1998; Beddington and Robertson 1999). In all vertebrates, the first overt appearance of L-R asymmetry occurs during early somitogenesis, with an initial rightward bending of the linear heart tube that presages the direction of cardiac looping. In the mouse, another early sign of laterality is the direction of embryonic turning that inverts the three primary germ layers of the embryo. Most morphological L-R asymmetry arises at later stages of organogenesis, when unilateral tissues such as the stomach are positioned on one side, or when bilateral paired tissues such as the lung form asymmetrically. Defects in this process of L-R specification can lead to highly pleiotropic effects, including L-R reversals of organ position (inverted situs), mirror image symmetry of bilaterally asymmetric tissues (isomerism), and/or random and independent occurrence of laterality defects in different tissues (heterotaxia).

Recent molecular genetic studies performed in chick, frog, zebrafish, and mouse systems have shown that tissue-specific laterality decisions are mediated by a pathway of regulatory genes that acts during gastrulation and early postgastrulation stages of embryogenesis. These studies have led to a conceptual pathway for L-R axis determination, in which an initial event that breaks L-R symmetry is believed to occur in or around the embryonic node and its derivatives. The resulting L-R positional information is transferred outward to the lateral plate mesoderm, where it is interpreted to generate the situs of individual tissues (for review, see Harvey 1998; Ramsdell and Yost 1998; Beddington and Robertson 1999; King and Brown 1999). Notably, several members of this regulatory pathway are themselves expressed in a...
L-R asymmetric pattern on the left side of the embryo, in particular the left lateral plate mesoderm.

Several genes in the L-R pathway have roles that appear evolutionarily conserved among vertebrates, including Nodal [Levin et al. 1995; Collignon et al. 1996; Lowe et al. 1996; Lustig et al. 1996; Lohr et al. 1997; Sampath et al. 1997; Rebagliati et al. 1998] and Lefty2 [Meno et al. 1996, 1997; Bisgrove et al. 1999; Thissie and Thissie 1999], which encode distant members of the transforming growth factor-β [TGF-β] superfamily, and are asymmetrically expressed in the left lateral plate mesoderm. Another conserved asymmetrically expressed gene is the Pitx2 homeobox gene, which has been proposed to represent a primary regulator of tissue-specific L-R laterality because it is expressed on the left side of many tissues [Logan et al. 1998; Piedra et al. 1998; Ryan et al. 1998; Yoshioka et al. 1998; Campione et al. 1999]. In contrast, there are several apparent differences between vertebrate systems that have complicated our understanding of the L-R pathway. For example, many genes that display transient asymmetry of expression in the chick are not asymmetrically expressed in the mouse, including activin βB, activin receptor IIA, and Sonic hedgehog [shh] (Harvey 1998; Ramsdell and Yost 1998; Beddington and Robertson 1999).

We show that L-R axis formation requires the evolutionarily conserved activity of members of the EGF-CFC gene family. The EGF-CFC family is comprised of mammalian Cryptic and Cripto, frog FRL-1, and zebrafish one-eyed pinhead (oep) and encodes extracellular proteins containing a divergent EGF-like motif and a novel cysteine-rich CFC motif [Shen et al. 1997; Zhang et al. 1998]. We find that targeted disruption of mouse Cryptic results in L-R laterality defects including randomization of abdominal situs, pulmonary right isomerism, and vascular heterotaxia, as well as randomized embryo turning and cardiac looping. In parallel studies, we show that partial rescue of oep mutant embryos by oep mRNA injection results in randomization of the direction of heart looping and location of the pancreas, revealing that loss of oep function leads to heterotaxia. Notably, in both Cryptic and oep mutant embryos, L-R asymmetric gene expression does not occur in the lateral plate mesoderm. Based on recent studies indicating that EGF-CFC proteins act as essential cofactors for Nodal [Gritsman et al. 1999], we propose that a signaling pathway mediated by nodal and EGF-CFC proteins is required for activation of L-R asymmetric gene expression in the lateral plate mesoderm.

Results

Targeted disruption of Cryptic

Previous mutational analyses have revealed that oep and Cripto have essential requirements prior to gastrulation [Schier et al. 1997; Ding et al. 1998; Gritsman et al. 1999], but it has been unclear if the later expression of EGF-CFC genes reflects a role in postgastrulation processes. In particular, oep is expressed in the lateral plate mesoderm and forebrain during early somitogenesis [Zhang et al. 1998], and Cryptic is expressed in the lateral plate mesoderm, node, notochordal plate, and prospective floor plate from head-fold stages through approximately the six to eight somite stage [Shen et al. 1997]. The expression of oep and Cryptic is symmetric in the lateral plate and precedes the asymmetric expression of genes such as Nodal/cyclops, Lefty2/antivin and Pitx2.

To determine the biological function of Cryptic, we performed targeted gene disruption. The Cryptic targeting construct should result in a null mutation, because it deleted most of the third exon and the entire fourth and fifth exons of the gene, which encode two-thirds of the mature protein including the central EGF and CFC motifs [Fig. 1a-c]. In addition, a second targeting construct that deleted the entire Cryptic coding region resulted in the identical homozygous mutant phenotype [Y.-T. Yan, S.M. Price, and M.M. Shen, unpubl.]. Homozygosity for the targeted Cryptic mutation resulted in neonatal lethality in the first 2 weeks after birth, apparently because of cardiac defects [see below]; to date, only five homozygotes [from >90] have survived past weaning. Our initial indication of a phenotypic defect in L-R laterality was that many newborn Cryptic homozygotes displayed a milk spot [corresponding to the stomach] on their right side, instead of the left (Fig. 1d).

L-R laterality defects in Cryptic mutant mice

To examine the L-R laterality defects of Cryptic homozygous mutant mice, we analyzed their gross anatomy at 18.5 days post coitum [dpc] and at neonatal stages [P0–P7] (Table 1). We found that Cryptic homozygotes displayed numerous laterality defects, including heterotaxia, randomization of organ situs, and isomerism of bilaterally asymmetric tissues. Thus, within the abdominal cavity, approximately half of the homozygotes (n = 22/49) displayed inverted situs of visceral organs including the stomach, spleen, and pancreas [Fig. 2a,b]. In contrast, all homozygous animals displayed asplenia or severe hyposplenias [Fig. 2c–e]; a significant proportion of homozygotes also displayed abnormal location or midline positioning of the liver (Table 1).

In the thoracic cavity, we found that all homozygotes showed right pulmonary isomerism [Fig. 2f,g]; this phenotype is correlated frequently with hyposplenias in human patients with laterality defects [Kosaki and Casey 1998]. Moreover, approximately half of the homozygotes (n = 24/50) displayed dextrocardia [cardiac apex pointing to the right] or mesocardia [pointing to the middle], as opposed to the normal levocardia (Fig. 2h–j). Regardless of cardiac situs, nearly all homozygotes displayed cardiac abnormalities, most notably transposition of the great arteries [Fig. 2k,l], as well as severe atrial septal defects [Fig. 2m–o]. Finally, we observed numerous random and uncorrelated laterality defects within the vasculature, consistent with heterotaxia (Table 1). For example, theazygos vein could be located on the left side (as it is in the wild type), on the right, or bilaterally (Fig. 2p–r).

The L-R laterality defects observed in neonatal Cryptic
EGF-CFC genes in left-right axis formation

homozygotes were paralleled by phenotypic defects observed in early embryogenesis. At 8.5–9.5 dpc, Cryptic homozygous embryos were indistinguishable from their wild-type littermates except for randomization of cardiac looping and embryo turning \( n = 21/45 \), with these two phenotypes highly correlated (Fig. 2s–u). Because Cryptic is expressed in the notochordal plate and prospective floor plate, and laterality defects are frequently associated with node and notochord defects [e.g., Danos and Yost 1996; Dufort et al. 1998; King et al. 1998; Melloy et al. 1998], we investigated potential axial midline defects by skeletal staining of homozygous neonates \( n = 6 \), histological sections at 10.5 dpc \( n = 3 \), and in situ hybridization with Shh, followed by sectioning \( n = 3 \). No evidence for axial midline defects was observed (data not shown).

**Absence of lateral L-R asymmetric gene expression in Cryptic mutant embryos**

To determine the basis for L-R patterning defects in Cryptic mutants, we performed in situ hybridization on gastrulation and early somite-stage embryos (up to 10 somites), using probes for *Nodal, Lefty1, Lefty2,* and *Pitx2,* which are asymmetrically expressed at these stages. First, we examined expression of *Lefty1* and *Lefty2,* which are asymmetrically expressed at 2–10 somites in the left prospective floor plate and left lateral plate mesoderm, respectively [Meno et al. 1997, 1998][Fig. 3a,b,e,f]. We found that Cryptic homozygous embryos lacked all expression of *Lefty1* \( n = 12 \) and *Lefty2* \( n = 9 \) at these stages [Fig. 3c,d,g,h]; however, an earlier phase of symmetric *Lefty2* expression in newly formed mesoderm during primitive streak stages was un-

**Table 1. Phenotype of Cryptic homozygous mice**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Number of mutant/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abdominal tissues</strong></td>
<td></td>
</tr>
<tr>
<td>Visceral situs</td>
<td>22/49 inverted (45%)</td>
</tr>
<tr>
<td>Spleen</td>
<td>49/49 asplenic/hyposplenic (100%)</td>
</tr>
<tr>
<td>Liver</td>
<td>11/27 abnormal (41%)</td>
</tr>
<tr>
<td><strong>Abdominal vasculature</strong></td>
<td></td>
</tr>
<tr>
<td>Branching of inferior vena cava</td>
<td>16/25 abnormal (64%)</td>
</tr>
<tr>
<td>Position of renal veins</td>
<td>4/19 left anterior (21%)</td>
</tr>
<tr>
<td><strong>Thoracic tissues</strong></td>
<td></td>
</tr>
<tr>
<td>Cardiac apex</td>
<td>18/50 dextrocardia, 6/50 mesocardia (48%)</td>
</tr>
<tr>
<td>Cardiac malformation</td>
<td>14/16 septal defects (88%), 11/11 transposition of great arteries (100%)</td>
</tr>
<tr>
<td>Atrial shape</td>
<td>23/34 right isomerism, 1/34 left (71%)</td>
</tr>
<tr>
<td>Lung bronchi</td>
<td>50/50 bilateral eparterial (100%)</td>
</tr>
<tr>
<td>Lung lobes</td>
<td>50/50 right isomerism (100%)</td>
</tr>
<tr>
<td><strong>Thoracic vasculature</strong></td>
<td></td>
</tr>
<tr>
<td>Aorta relative to pulmonary artery</td>
<td>44/50 aorta ventral, 5/50 adjacent (98%)</td>
</tr>
<tr>
<td>Aortic arching</td>
<td>12/29 right (41%)</td>
</tr>
<tr>
<td>Position of azygos vein</td>
<td>5/30 right, 6/30 bilateral (37%)</td>
</tr>
<tr>
<td>Position of inferior vena cava</td>
<td>4/33 left, 19/33 bilateral (70%)</td>
</tr>
</tbody>
</table>

Phenotypes were scored in mice at 18.5 dpc \( n = 6 \) and in neonates at <1 week of age \( n = 44 \). Not all phenotypes were scored in each mouse. Unless indicated otherwise in the text, the occurrences of these phenotypes were not noticeably correlated with each other.

**Figure 1.** Targeted disruption of Cryptic. (a) Homologous recombination with the targeting vector results in deletion of exons 4 and 5, as well as most of exon 3; exons are shown as boxes, with the coding region in dark gray; [A] AvrII; [E] EcoRI; [N] NheI; [S] SmaI; [X] XbaI. (b) Southern blotting using the 5′-flanking probe. A detects an 18-kb EcoRI fragment from the wild-type genomic locus and an 8.5-kb fragment from the targeted allele in progeny of F1 heterozygous intercrosses; dashes (left) indicate positions of markers at 5 and 10 kb. (c) PCR analysis of visceral yolk sac DNA from 7.5-dpc embryos, showing amplification of an 860-bp band corresponding to Cryptic and a 735-bp fragment corresponding to neo; markers at 615 and 861 bp are indicated as above. (d) Neonatal mice with milk spots on the right side (-/-) and left side [WT].
Figure 2. L-R laterality defects in Cryptic null mutants. (a–r) Ventral views of neonatal mice, in all panels, left (L) and right (R) are as indicated. Abdominal cavity of wild type (a) and mutant (b) with inverted situs and hypoplasia. Stomach and spleen from wild type (c), mutant with normal situs (d), and mutant with inverted abdominal situs (e). Heart and lung lobes of wild type (f) and mutant (g) with right pulmonary isomerism. Heart positions of wild type with normal levocardia (h), and mutants with mesocardia (i) and dextrocardia (j), note correlation with altered size of the atrial chambers. High-power view of cardiac arterial relationships. In the wild type (k), the aorta is dorsal to the pulmonary artery and connects to the left ventricle; in the mutant (l), the aorta is ventral and connects to the right ventricle, as shown following injection of blue dye into the right ventricle, consistent with transposition of the great arteries. Sections through hearts of wild type (m), and two mutants (n,o) that show an atrial septal defect. Position of the azygos vein and direction of aortic arching. In the wild type (p), the azygos vein is located on the left, and the aorta arches leftward; in the mutant (q), the azygos vein crosses over to the right and the aorta arches rightward; in the mutant (r), there is a bilateral azygos vein while the aorta arches leftward. (s) Lateral views of 8.5-dpc embryos, showing altered direction of embryo turning in the mutant. (t,u) Ventral views of 10-somite-stage embryos, with arrows indicating direction of cardiac looping in the wild type (t) and Cryptic mutant (u). (v) accessory lobe; (ao) aorta; (asd) atrial septal defect; (az) azygos vein; (cl) caudal lobe; (cr) cranial lobe; (ht) heart; (la) left atrium; (ll) liver; (ll) left lung; (lv) left ventricle; (ml) medial lobe; (pa) pulmonary artery; (ra) right atrium; (rv) right ventricle; (sp) spleen; (st) stomach; (tb) tailbud.

Finally, we examined expression of the homeobox gene Pitx2, which is found symmetrically in Rathke’s pouch and asymmetrically in the left lateral plate mesoderm and left foregut endoderm from six to eight somites continuing through 9.5 dpc [Ryan et al. 1998; Yoshioka et al. 1998] (Fig. 3i, l). In Cryptic mutants at 8.5 and 9.5 dpc (n = 16), Pitx2 expression was still observed in Rathke’s pouch but not in the asymmetric domains (Fig. 3k, l).

Finally, we examined expression of Nodal, which is found at the lateral boundaries of the node at head-fold and early somite stages, with a transient phase of L-R asymmetry at four to eight somites, and in the left lateral plate mesoderm at approximately two to eight somites [Collignon et al. 1996; Lowe et al. 1996] (Fig. 3m, n). In Cryptic mutants (n = 41), Nodal expression was observed at the lateral boundaries of the node but was never detected in the lateral plate mesoderm (Fig. 3o, p). Notably, the markedly asymmetric expression of Nodal at the edges of the node at four to eight somites was still observed in the Cryptic mutant embryos (n = 29; Fig. 3q, s). Thus, our in situ hybridization results indicate that L-R
laterality has been initiated within the node but not in the lateral plate mesoderm.

Heterotaxia in oep mutant fish

To determine if the function of EGF–CFC genes in L-R determination is conserved in vertebrates, we studied the role of the zebrafish oep gene. Previous studies have shown that oep is required for formation of mesoderm, endoderm, prechordal plate, and ventral neuroectoderm, correlating with the expression of oep in the progenitors of these cell types (Schier et al. 1997; Zhang et al. 1998; Gritsman et al. 1999). During somitogenesis, oep is also expressed in the left and right lateral plate, where progenitors of the heart and other organs are located in wild-type embryos (Serbedzija et al. 1998; Zhang et al. 1998). The potential role of oep in these territories cannot be analyzed in oep mutants, because mutant embryos lack...
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Table 2. More than 81% (48/59) of oep mutant embryos that display abnormal heart asymmetry during embryogenesis survive to adulthood, demonstrating that mRNA injection rescues the development and function of all essential organs. Notably, there was no correlation between abnormal heart asymmetry and the location of the pancreas, revealing that loss of oep function leads to heterotaxia.

Absence of L-R asymmetric gene expression in oep mutants

To determine the onset of the L-R patterning defect in oep mutants, we performed in situ hybridization on somite-stage embryos using probes for cyclops, antivin (a member of the lefty family), and pitx2, which are all asymmetrically expressed in the lateral plate mesoderm (Rebagliati et al. 1998; Campione et al. 1999; Thisse and Thisse 1999). Analogous to Cryptic mouse mutants, we never detected the normal asymmetric expression of these markers, despite wild-type expression in other regions of the embryo (Fig. 5). Importantly, asymmetric expression is not initiated, revealing a role for oep in the induction of lateral plate asymmetry.

Discussion

Our comparative mutational analyses have shown that homozygous Cryptic null mutant mice and partially rescued oep mutants show cyclopia and absence of trunk muscle (Gritsman et al. 1999). To circumvent this limitation, we examined the phenotype of maternal-zygotic oep [MZoep] embryos whose early defects were rescued by oep mRNA injection (Fig. 4). Injected mRNA is present throughout gastrulation (data not shown) and is sufficient to completely rescue the formation of endoderm, mesoderm, axial midline, and ventral neuroectoderm (Fig. 4c-f), but is apparently insufficient to complement the loss of oep activity at later stages. Using morphological criteria and marker gene expression, we found that heart and pancreas form, but that the direction of heart looping and the location of the pancreas are randomized with respect to the L-R axis of the embryo (Fig. 4c-f).

Table 2. Direction of heart looping and location of pancreas in wild-type and oep mutants

<table>
<thead>
<tr>
<th>Genotype: oep/+ [++] female x +/- male</th>
<th>Genotype: oep-/- female x +/- male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected mRNA: lacZ</td>
<td>Injected mRNA: oep</td>
</tr>
<tr>
<td>Total no. embryos: 96</td>
<td>Total no. embryos: 108</td>
</tr>
<tr>
<td>Heart (MF20)</td>
<td></td>
</tr>
<tr>
<td>L 51 (48.1%)</td>
<td>R 51 (48.1%)</td>
</tr>
<tr>
<td>M 26 (24.5%)</td>
<td>M 26 (24.5%)</td>
</tr>
<tr>
<td>R 24 (22.7%)</td>
<td>R 24 (22.7%)</td>
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</table>

Figure 4. Heart looping and location of the pancreas in oep mutants. Ventral [a,c,e] and dorsal views [b,d,f] of embryos upon immunohistochcmistiy with MF20 antibody [Bader et al. 1982] and RNA in situ hybridization with insulin probe [Milewski et al. 1998], anterior is up. [a,b] Wild-type embryo; [c,d and e,f] two examples of maternal-zygotic oep mutant embryos rescued by injection of oep mRNA. Note the normal development of eyes and trunk muscle [d,f] in rescued embryos [maternal-zygotic oep mutants show cyclopia and absence of trunk muscle (Gritsman et al. 1999)]. The arrow indicates heart looping from the atrium [weaker staining, posteriorly] to the ventricle [stronger staining, anteriorly]; note right looping in a and left looping in c and e. The arrowhead indicates position of pancreas on right [b,d] or left [f] side. We note that the direction of cardiac looping in rescued mutants did not significantly affect their survival to adulthood [50/70 (71%) of right-looping embryos, 46/53 (87%) of left-looping embryos, and 2/6 (33%) of nonlooping embryos survived].

Table 2. Direction of heart looping and location of pancreas in wild-type and oep mutants

<table>
<thead>
<tr>
<th>Pancreas (insulin)</th>
<th>Heart (MF20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>M</td>
</tr>
<tr>
<td>R 51 (48.1%)</td>
<td>20 (18.9%)</td>
</tr>
<tr>
<td>M 26 (24.5%)</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>L 24 (22.7%)</td>
<td>24 (22.7%)</td>
</tr>
</tbody>
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</tr>
<tr>
<td>L 24 (22.7%)</td>
<td>24 (22.7%)</td>
</tr>
</tbody>
</table>
cued oep mutant fish both display highly penetrant L-R heterotaxia defects. Notably, in Cryptic as well as oep mutant embryos, Nodal, Lefty2/antivin, and Pitx2 are not expressed in the lateral plate mesoderm, indicating that EGF-CFC activity is essential for asymmetric gene expression in the lateral mesoderm. Taken together, our findings with oep mutant fish are analogous to those for Cryptic mutant mice, and establish an evolutionarily conserved requirement for EGF-CFC genes in the establishment of L-R asymmetry in vertebrates. Interestingly, this evolutionary conservation of EGF-CFC activity in the L-R pathway markedly contrasts with the apparent non-conserved roles of Fgf8 and Shh in the mouse and chick (Meyers and Martin 1999).

Essential function of EGF-CFC genes in L-R axis specification

Our results can be readily integrated with a general pathway for L-R axis determination in which initial L-R symmetry is broken in or around the node, and subsequent L-R positional information is transferred to the lateral plate mesoderm (Levin et al. 1995; Logan et al. 1998; Pagan-Westphal and Tabin 1998; Bedдинton and Robertson 1999). Given the requirement of oep activity for nodal signaling in zebrafish and the functional conservation of EGF-CFC proteins (Gritsman et al. 1999), we propose that Cryptic and oep are essential for Nodal signaling in L-R axis specification. Our findings indicate that EGF-CFC activity is required prior to the activation of L-R asymmetric gene expression in the periphery and may be involved in events downstream from an initial process that breaks L-R symmetry.

Specifically, our results are consistent with two possible models for EGF-CFC function in L-R axis formation (Fig. 6). In the first model, Cryptic/oep would be required in the lateral plate mesoderm to mediate the response to an asymmetric 'left'-determining signal emanating from the node (Fig. 6a,b). This signal might correspond to Nodal itself, as we have shown previously that EGF-CFC proteins are required for cells to respond to Nodal signals (Gritsman et al. 1999). In this scenario, Nodal signaling from the node or its derivatives cannot be received due to the absence of EGF-CFC activity in the lateral plate.

In the second model for EGF-CFC function, Cryptic/oep would be required at an earlier stage in the node or its derivatives for the generation or propagation of an asymmetric signal, which could either correspond to Nodal itself or be dependent on Nodal signaling (Fig. 6c). Defects in axial midline structures often result in L-R laterality defects and alterations in asymmetric gene expression, as seen for mouse mutations in no turning, HNF-3β, Brachyury, and SIL (Dufort et al. 1998; King et al. 1998; Melloy et al. 1998; Izraeli et al. 1999) and for zebrafish mutations in no tail or floating head (Danos and Yost 1996; Chen et al. 1997). Although there are no apparent structural defects in the node or its derivatives in Cryptic and partially rescued oep mutants, absence of EGF-CFC activity might result in a block in Nodal signaling in the axial midline, indirectly leading to defects in the lateral plate (Fig. 6c). Of course, these models are not mutually exclusive, and Cryptic/oep may act in both the node and lateral plate. In either case, EGF-CFC activity would play an essential role in transferring L-R positional information from the node to the periphery, resulting in asymmetric Nodal and Lefty2/antivin expression in the lateral plate mesoderm, asymmetric Left1 expression in the mouse floor plate, and subsequent asymmetric Pitx2 expression, ultimately leading to specification of individual organ situs.
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**Figure 6.** Schematic model for EGF-CFC function in L-R axis formation. (a) In wild-type embryos, a asymmetric signal emanating from the node activates expression of Nodal/cyclops and Lefty2/antivin in the left lateral plate mesoderm, as well as Lefty1 in the left prospective floor plate, leading to subsequent activation of Pitx2 and specification of organ situs. The node-derived signal could correspond to Nodal itself or to a hypothesized factor downstream of Shh signaling that conveys L-R positional information in the chick (Pagan-Westphal and Tabin 1998). (b) In this model, the response to the asymmetric node-derived signal is mediated by Cryptic/oep, which is symmetrically expressed in the lateral plate mesoderm. In the absence of EGF-CFC activity, the lateral plate and prospective floor plate do not respond to the node-derived signal, and asymmetric gene expression fails to occur, resulting in subsequent L-R laterality defects. (c) Here, Cryptic/oep is required to mediate a Nodal activity that is downstream of an initial L-R symmetry-breaking event in or around the node. As a consequence, L-R laterality is specified around the node but fails to be propagated to the lateral plate mesoderm.

**Cryptic mutants as a model for right isomerism/asplenia syndrome**

In humans, the proper L-R situs of the visceral tissues is critical for their morphogenesis and/or physiological function, particularly in the cardiovascular system. In particular, children born with severe heterotaxia generally die shortly after birth, usually due to severe cardiac defects. In many cases, laterality defects in humans that result in heterotaxia can be classified into two primary categories: right isomerism associated with asplenia/hypoplasia and, conversely, left isomerism associated with polysplenia (Goldstein et al. 1998; Kosaki and Casey 1998). Our studies show that Cryptic mutant mice recapitulate many features of the right isomerism/asplenia syndrome, suggesting that the Cryptic mutant mice may represent a model for a major category of human L-R laterality defects.

**Interaction of EGF-CFC genes with the Nodal signaling pathway**

In contrast to the phenotype reported here for oep, mutations in the zebrafish nodal gene cyclops do not result in a significant incidence of heart looping defects (Chen et al. 1997). These differing laterality phenotypes of oep versus cyclops mutants may be due to redundant functions of zebrafish nodal-related genes in L-R axis determination. Moreover, a direct requirement for mouse Nodal in L-R patterning has also been difficult to establish, due to the early embryonic lethality of Nodal mutants, which precludes analysis of later defects. Nonetheless, the L-R phenotypes of Cryptic and oep mutants, together with the phenotypes of Nodal"/"; HNF-3B"/" and Nodal"/"; Smad2"/" mutants (Collignon et al. 1996; Nomura and Li 1998), strongly suggest that Nodal signals are essential for L-R axis specification.

Although the Nodal signaling pathway has not been analyzed at the biochemical level, loss- and gain-of-function studies in mouse, frog, and fish suggest that during gastrulation Nodal signals may act via activin-like receptors (Hemmati-Brivanlou and Melton 1992; Armes and Smith 1997; Chang et al. 1997; New et al. 1997; Oh and Li 1997; Gu et al. 1998; Gritsman et al. 1999; Meno et al. 1999) and the transcription factor Smad2 (Baker and Harland 1996; Graff et al. 1996; Nomura and Li 1998; Waldrip et al. 1998; Weinstein et al. 1998). During germ-layer formation, Nodal signaling has also been shown to be dependent on EGF-CFC activity (Gritsman et al. 1999) and to be antagonized by members of the Lefty family (Bisgrove et al. 1999; Meno et al. 1999; This and This 1999). Therefore, it is thought that during gastrulation, Nodal signals are dependent on EGF-CFC proteins to activate activin-like receptors and Smad2, leading to the induction of Lefty genes and the attenuation of Nodal signaling.

Comparison of the L-R phenotypes of Cryptic and oep mutants with the defects found in Lefty1 and ActRIB mutant mice extends this model to L-R axis determination. These differing laterality phenotypes of oep versus cyclops mutants may result from the involvement of additional factors in L-R axis determination. Moreover, a direct requirement for mouse Nodal in L-R patterning has also been difficult to establish, due to the early embryonic lethality of Nodal mutants, which precludes analysis of later defects. Nonetheless, the L-R phenotypes of Cryptic and oep mutants, together with the phenotypes of Nodal"/"; HNF-3B"/" and Nodal"/"; Smad2"/" mutants (Collignon et al. 1996; Nomura and Li 1998), strongly suggest that Nodal signals are essential for L-R axis specification.
mutants display right pulmonary isomerism and lack asymmetric gene expression in the lateral plate mesoderm. These opposing phenotypes support the notion that Lefty1 acts by antagonizing EGF-CFC dependent Nodal activity during L-R determination. Secondly, the phenotype of Cryptic mutants superficially resembles that of ActRIIB mutant mice, which display right pulmonary isomerism and severe cardiac defects (Oh and Li 1997). Moreover, although Smad2 homozygotes display early embryonic lethality due to defective specification of the anteroposterior [AP] axis [Nomura and Li 1998; Waldrip et al. 1998; Weinstein et al. 1998], a significant percentage of Nodal<sup>−/−</sup>; Smad2<sup>−/−</sup> compound heterozygotes display L-R laterality defects [Nomura and Li 1998], which are similar to those of Cryptic mutants. The greater severity of the laterality defects in Cryptic mice relative to those of ActRIIB mutants may reflect the ability of Nodal in conjunction with EGF-CFC proteins to signal through a type II receptor that is partially redundant with ActRIIB, perhaps ActRIIA (also known as ActRII). In summary, these findings indicate that Nodal signaling during L-R development is mediated by EGF-CFC proteins, activin receptors, and Smad2.

**Conservation of EGF-CFC function in embryonic axis formation**

The phenotypes of Cripto and Cryptic mutations in mice bear remarkable similarity to those of mutant zebrasfish with different timing of oep activity. Specifically, complete removal of both maternal and zygotic oep activity [MZoep mutants] results in loss of head and trunk mesoderm, endoderm, and an incorrectly positioned AP axis [Gritsman et al. 1999], a phenotype similar to that of Cripto mutant mice [Ding et al. 1998]. Conversely, restoration of early oep activity to MZoep embryos by oep mRNA injection rescues these defects, but the insufficient persistence of injected mRNA results in a subsequent L-R laterality defect that strongly resembles the phenotype of Cryptic mutants. Taken together, our results indicate that a Nodal and EGF-CFC signaling pathway is essential for both the AP and L-R axes in vertebrates, with the dual role for oep in both processes in fish being divided between the related genes Cripto and Cryptic in mice.

**Materials and methods**

**Gene targeting**

A murine Cripto cDNA was used to screen a λFIXII library constructed from 129Sv/J genomic DNA [Stratagene], resulting in the isolation of a 21-kb genomic clone containing the entire coding region. To construct a targeting vector for Cripto, a 3.5-kb Xbal-Smal 5′ flank was subcloned into the Xbal-Smal sites of pTKLNL [Mortensen 1999], followed by subcloning of a 5.0-kb Smal-Natl 3′ flank, such that the PGK-neo and PGK-tk cassettes are in the opposite transcriptional orientation to Cripto. Targeting was performed using TCI ES cells [Deng et al. 1996], with targeted clones obtained at a frequency of 5% [4/88].

ES cell culture and blastocyst injection were performed as described previously [Ding et al. 1998]. Chimeric males obtained following blastocyst injection were bred with Black Swiss females [Taconic], and germ-line transmission was obtained from one targeted ES clone; two independent lines were also derived using a different targeting vector [Y.-T. Yan, S.M. Price, and M.M. Shen, unpubl.]. These targeted Cryptic mutations have been maintained through backcrossing with outbred Black Swiss mice; the phenotype appears similar in each line. In addition, the homozygous phenotype appears similar in a hybrid 129/SvEvTac-C57BL/6j strain background.

**Mouse genotyping and phenotypic analysis**

Genotyping was performed by Southern blotting or by PCR using genomic DNA prepared from tails or embryonic visceral yolk sac. Primers for genotyping were as follows: for wild-type Cryptic, 5′GGAGATGGTGCCAGAGAAGTCAGC3′ and 5′AATAGCGACGGGACACGGAAACAC3′; for neo, 5′CTGGCGCGCTGTTCTCCTCTTCTC3′ and 5′ACACCCCGGCGCCACAGTCG3′. The presence of cardiac septal defects and transposition of the great arteries was scored by injection of bromphenol blue dye into the right ventricle [Oh and Li 1997], and ventriculoarterial alignment was confirmed by histological sectioning. Cardiac histology was performed by hematoxylin-eosin staining of paraffin sections, with attention given to L-R orientation of sections. Whole-mount in situ hybridization to mouse embryos was performed as described [Ding et al. 1998], using probes for murine Lefty1 [Menlo et al. 1997], Lefty2 [Menlo et al. 1997], Nodal [Lowe et al. 1996], and Pitx2 [Lancet et al. 1999].

**Zebrafish genetics and phenotypic analysis**

Homozygous oep<sup>−/−</sup> (oep<sup>−/−</sup>) mutants were obtained by rescue of homozygous oep<sup>−/−</sup> (oep<sup>−/−</sup>) embryos with oep mRNA [Zhang et al. 1998; Gritsman et al. 1999]. To rescue the early patterning defects of oep mutants, maternal-zygotic oep<sup>−/−</sup> (oep<sup>−/−</sup>) embryos were injected with 25–50 pg of oep mRNA at the one-to-four-cell stage. Heart looping was scored in live embryos and by immunohistochemistry using the M20 antibody [Bader et al. 1982] that recognizes a myosin heavy chain. Embryos were then processed for in situ hybridization using an insulin antisense RNA probe [Milewski et al. 1998]. Whole-mount in situ hybridization for cyclops, antivin, and Pitx2 was performed as described [Zhang et al. 1998]. Zebrafish pitx2 was cloned by screening of a CDNA library kindly provided by B. Appel and J. Eisen, University of Oregon, Eugene with a PCR-amplified pitx2 homeobox probe [R.D. Burdine; A.F. Schier, and W.S. Talbot, GenBank accession nos. AF156905 and AF156906].

**Acknowledgments**

We thank Anukampa Barth, Jacques Drouin, Hiroshi Hamada, Yoshiyuki Imai, Michael Kuehn, Rick Mortensen, Cliff Tabin, Bernard Thiese, Christine Thiese, and Steve Wilson for gifts of probes and reagents. We also thank Nishita Desai, Rory Feeney, Elizabeth Heckscher, and Magdalena Michalski for technical assistance. We are particularly grateful to Cory Abate-Shen, Robert Cardiff, and Cliff Tabin for helpful discussions and comments on the manuscript. This work was supported by post-doctoral fellowships from the American Heart Association [J.D.] and Damon Runyon-Walter Winchell Cancer Research Fund [R.D.B.], and by grants from the National Science Foundation [M.M.S.], the American Heart Association [M.M.S.], the U.S.
References


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