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Chick CFC Controls Lefty1 Expression in the Embryonic Midline and Nodal Expression in the Lateral Plate

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Members of the EGF-CFC family of proteins have recently been implicated as essential cofactors for Nodal signaling. Here we report the isolation of chick CFC and describe its expression pattern, which appears to be similar to Cfc1 in mouse. During early gastrulation, chick CFC was asymmetrically expressed on the left side of Hensen's node as well as in the emerging notochord, prechordal plate, and lateral plate mesoderm. Subsequently, its expression became confined to the heart fields, notochord, and posterior mesoderm. Implantation experiments suggest that chick CFC expression in the lateral plate mesoderm is dependent on BMP signaling, while in the midline its expression depends on an Activin-like signal. The asymmetric expression domain within Hensen's node was not affected by application of FGF8, Noggin, or Shh antibody. Implantation of cells expressing human or mouse CFC2, or chick CFC on the right side of Hensen's node randomized heart looping without affecting expression of genes involved in left–right axis formation, including SnR, Nodal, Car, or Pitx2. Application of antisense oligodeoxynucleotides to the midline of Hamburger–Hamilton stage 4-5 embryos also randomized heart looping, but in contrast to the overexpression experiments, antisense oligodeoxynucleotide treatment resulted in bilateral expression of Nodal, Car, Pitx2, and NKX3.2, whereas Lefty1 expression in the midline was transiently lost. Application of the antisense oligodeoxynucleotides to the lateral plate mesoderm abolished Nodal expression. Thus, chick CFC seems to have a dual function in left–right axis formation by maintaining Nodal expression in the lateral plate mesoderm and controlling expression of Lefty1 expression in the midline territory.

Key Words: **chicken; embryo; EGF-CFC; Cfc1; Cfc2; nodal; Lefty1; left–right axis; asymmetric gene expression; heart looping.**

INTRODUCTION

Vertebrates develop numerous left–right (L–R) asymmetries in both visceral and thoracic organs from an initially bilaterally symmetric embryo. However, no common theory is presently available as to how the lateral symmetry is broken in different organisms and as to how the L–R axis is established with respect to the dorsoventral and anterioposterior axes. Irrespective of the exact mechanisms, which initially establishes L–R asymmetry in vertebrates, the node is a target for lateralization. In the chick, L–R asymmetry of Hensen's node is determined by the surrounding tissue between HH stages 4 and 5 (Pagán-Westphal and Tabin, 1998). A cascade has been identified which transfers L–R asymmetry from the node to the lateral plate (Burdine and Schier, 2000; Capdevilla *et al.*, 2000). Expression of Activin βB and its receptor ActRIIA is restricted to the right side of Hensen's node (Levin *et al.*, 1995, 1997). Activin controls the right-sided expression of FGF8 (Boettger *et al.*, 1999) and N-Cadherin (Garcia-Castro *et al.*, 2000). While

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N-Cadherin might be involved in the control of cell migration through the node, FGF8 is involved in suppressing Nodal expression in the right lateral plate mesoderm. Shh is initially expressed symmetrically in the node and becomes lateralized through inhibition on the right side by Activin (Levin *et al.*, 1995). Shh activates Nodal in a small domain lateral to the node on the left side. Subsequent transfer of positional information to the lateral plate appears to require paraxial mesoderm (Pagán-Westphal and Tabin, 1998) and the Cerberus homologue Caronte (Car) (Rodriguez Esteban *et al.*, 1999; Yokouchi *et al.*, 1999; Zhu *et al.*, 1999). Car induces Nodal expression by binding to and thereby inhibiting BMP2 present in the lateral mesendoderm (Andrée *et al.*, 1998). The bicoid-type homeobox gene Pitx2 acts downstream of Nodal in the left lateral plate mesoderm (LPM). Both overexpression of Pitx2 in the chick embryo and null mutation in mouse cause laterality defects (Campione *et al.*, 1999; Logan *et al.*, 1998; Ryan *et al.*, 1998; Lu *et al.*, 1999). Other transcription factors downstream of Nodal in the LPM include the homeobox gene NKX3.2 on the left side and the zinc finger gene SnR on the right (Isaac *et al.*, 1997; Schneider *et al.*, 1999). While the role of NKX3.2 in the establishment of L–R asymmetry is presently unclear, SnR is involved in suppressing Pitx2 expression on the right side (Patel *et al.*, 1999).

An intact midline is crucial for the development and maintenance of L–R identity. Embryological manipulations in frog (Danos and Yost, 1996) and zebrafish mutants with abnormal notochord development such as no tail and floating head (Rebagliati *et al.*, 1998; Sampath *et al.*, 1998), and the mouse mutants No turning and SIL (Izraeli *et al.*, 1999; Melloy *et al.*, 1998) show randomized heart looping and bilateral Nodal expression. These results have led to the proposal that the midline serves as a barrier to block the spreading of signals from the left to the right side. Lefty1 might act as a midline barrier molecule. Lefty1 has been shown to be expressed in the notochord and floorplate and Lefty1 null mutants develop left isomerism (Meno *et al.*, 1998). Lefty1 has been proposed to prevent Nodal signaling from spreading across the midline by binding to the Nodal receptor as a competitive inhibitor (Meno *et al.*, 1998; Rodriguez Esteban *et al.*, 1999).

One-eyed pinhead (oep) in zebrafish, FRL in *Xenopus*, and Cfc1 and -2 (formerly known as Cryptic and Cripto) in mouse are members of the EGF-CFC family that encode extracellular proteins sharing an aminoterminal signal peptide, an epidermal growth factor (EGF)-like motif, a cysteine-rich domain (CFC motif), and a carboxyterminal hydrophobic region which in the case of CFC2 is required for glycosylphosphatidylinositol (GPI) linkage to the cell membrane (Minchiotti *et al.*, 2000; Salomon *et al.*, 1999; Shen and Schier, 2000). Genetic studies in zebrafish and mouse have identified EGF-CFC peptides as essential cofactors for Nodal signaling (Gritsman *et al.*, 1999). Embryos lacking both maternal and zygotic activity of oep display a complete disruption of axial structures, as observed in double mutants for the Nodal factors cyclops and squint (Gritsman *et al.*, 1999). In the mouse, Cfc2 mutants lack a primitive streak, embryonic mesoderm and display an incorrectly positioned A/P axis similar to the phenotype observed in Nodal mutants (Ding *et al.*, 1998). Targeted disruption of mouse Cfc1 results in L–R laterality defects, including randomization of heart looping and loss of expression of Nodal, Lefty2, and Pitx2 in the LPM and Lefty1 in the left floorplate (Gaio *et al.*, 1999; Yan *et al.*, 1999). Maternal-zygotic oep mutants whose early defects in mesoderm and endoderm formation were rescued by oep mRNA injection also display heterotaxia and lack expression of L–R determinants, suggesting conserved functions of oep and Cfc1 in L–R axis formation (Shen and Schier, 2000).

In this report we have analyzed the function of chick CFC, a novel member of the EGF-CFC family. Chick CFC exerts L–R asymmetric expression in Hensen's node during the critical period of L–R axis specification. In addition, chick CFC is expressed in LPM and in the forming notochord. Overexpression of mouse or human CFC2 and chick CFC results in aberrant cardiac looping without altering the expression of L–R axis determinants, such as Nodal, Car, SnR, and Pitx2. In contrast, localized antisense oligodeoxynucleotide treatment of Hensen's node or LPM revealed essential functions of chick CFC for the expression of Lefty1 in the midline and of Nodal in the LPM.

MATERIALS AND METHODS

Isolation of Chick CFC

A spotted cDNA library of HH stage 3–6 chicken embryos (Resource Center of the German Human Genome Project, Library No. 573) was screened at low stringency using a full-length probe of mouse Cfc2 as previously described (Andrée *et al.*, 1998). Hybridizing clones were further analyzed by Southern blot analysis and sequenced. A single cDNA was identified, which was named chick CFC (GenBank Accession No. AF282984). Chick CFC was found to be identical in sequence to the previously published clone chick cripto (GenBank Accession No. AF282984; Colas and Schoenwolf, 2000).

Whole-Mount In Situ Hybridization

Whole-mount *in situ* hybridizations were carried out as described (Andrée *et al.*, 1998). For the detection of chick CFC, a 1-kb *Hin*dIII/*Sal*I fragment was used. For expression analysis of other marker genes the probes were Nodal, 500 bp (Levin *et al.*, 1995); Sonic hedgehog, 1.6 kb (Riddle *et al.*, 1993); Caronte, 600 bp (Rodriguez Esteban *et al.*, 1999); FGF8, 0.8 kb (Crossley *et al.*, 1996); SnR, 1.8 kb (Isaac *et al.*, 1997); Pitx2, 1 kb (St Amand *et al.*, 1998); Lefty1, 1.1 kb (Ishimaru *et al.*, 2000); NKX3.2, 1.2 kb (Schneider *et al.*, 1999).

Antisense Oligonucleotides

The antisense oligodesoxynucleotides (antisense ODN; Eurogentec, Cologne) were targeted against nt 130–147 of the chick CFC cDNA (antisense1: 5′-A*T*G*TTTTCGCCAGAA*C*A*T-3′; the asterisks indicate phosphorothioate modifications of the phosphate backbone) and nt 234–253 (antisense2: 5′-GCGCTGTGTCGTTG-AAATTT-3′). As controls, ODNs were synthesized corresponding to the reverse sequence of antisense1 (control1: 5′-T*A*C*AAAA GCG-

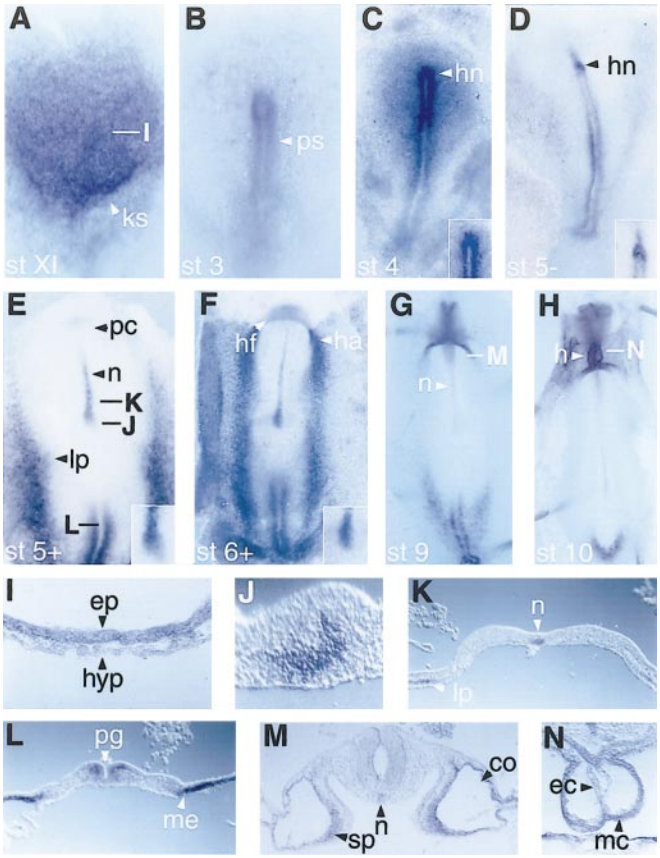


FIG. 1. Expression of chick CFC in developing chicken embryos. Chick CFC was detected by whole-mount *in situ* hybridization with a full-length chick CFC antisense cRNA probe. Ventral views of stained embryos at (A) EGK stage XI, (B) HH stage 3, (C) HH stage 4, (D) HH stage 5, (E) HH stage 6, (F) HH stage 7, (G) HH stage 8, (H) HH stage 10. (I–N) are transverse sections through the embryos shown in (A, D, E, G, H) as indicated. Plane of sectioning is indicated in the individual panel. co, coelom; ec, endocard; ep, epiblast; ks, Köller's sickle; ps, primitive streak; h, heart; ha, heart anlage; hf, head fold; hn, Hensen's node; lp, lateral plate; mc, myocard; me, mesendoderm; n, notochord; pc, prechordal plate; pg, primitive groove; sp, splanchnic mesoderm.

GTCTT*G*T*A-3′) and antisense2 (control2: 5′-CGCGACACAG-CAACTTTAAA-3′), respectively. In order to visualize the spatial distribution of the ODNs after application to the embryo, a 5′-FITC-labeled ODN identical to antisense2 was used. ODNs were dissolved at 2 mM in water and diluted with a 20% (w/v) solution of Pluronic F-127 (Sigma, Steinheim) in Pannett–Compton saline to give a final concentration of 20 μM.

Fertilized chicken eggs (Charles River, Sulzfeld, Germany) were incubated at 38°C with 60% humidity. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) and placed into New culture as previously described (New, 1955; Schlange *et al.*, 2000). Experiments were carried out on HH stage 4–5 embryos. The embryos were removed from the incubator and left at room temperature for 15 min before approximately 2 μl of the ODN–Pluronic gel mixture was applied to the node or the lateral plate region. Embryos were cultured for a variable length of time and then fixed and processed for *in situ* hybridization.

Implantation of Cell Aggregates or Beads

CHO.B3.A4 cells expressing *Xenopus* Noggin and CHO dhfr- cells, which were used as controls, were cultured as previously described (Schlange *et al.*, 2000). Q2bn cells producing BMP2 and control cells were cultured as previously described (Andrée *et al.*, 1998). Human 293T embryonal kidney cells expressing mouse Cfc2 and Cfc2 ΔC lacking the hydrophobic C-terminal stretch required for membrane localization and GPI linkage (Minchiotti *et al.*, 2000) and human SiHa cervical carcinoma cells expressing human CFC2 (S-CR1) and control cells (S-pCI) (Ebert *et al.*, 1999) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) FCS. A 1-kb *Hin*dIII fragment of chick CFC was subcloned into pcDNA3.1+ (Invitrogen) and transfected into COS-7 cells by calcium phosphate precipitation. A stable cell line expressing chick CFC was isolated after neomycine selection. Cell aggregates for implantation were produced by trypsinizing confluent 90 mm ϕ culture dishes and subsequently culturing the cells in bacteriological petri dishes. After 1 or 2 days the cells formed cell aggregates suitable for implantation.

Heparin acryl beads (Sigma) were incubated for 30 min with 100 ng/μl mouse FGF8b (R&D Systems, Wiesbaden), or 50 ng/ml human recombinant Activin A, Lot No. 15365-36(1), which was obtained through the National Hormone and Pituitary Program. AG 1-X2 anion exchange beads (Bio-Rad) were incubated for 30 min with 2 mM SU 5402 (Calbiochem) in DMSO. Beads were washed twice in DMSO and once in PBS before being implanted into the embryo. The hybridoma 5E1 against Shh (Ericson *et al.*, 1996) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa. Shh antibody secreted by the hybridoma cells was concentrated by protein G sepharose chromatography and used as a 1 mg/ml solution in PBS containing 1% BSA. AffiGel blue beads (Bio-Rad) were soaked for 1 h with 0.5 mg/ml human Activin RIIA extracellular domain fused to human IgG_1 Fc (R&D Systems). Eggs (White Leghorn, Charles River, Extertal) were incubated for approximately 24 h until they had reached HH stage 4–6 and placed into New culture (New, 1955). For control purposes the different beads were soaked in vehicle solution containing 1% BSA. A tungsten needle was used to make small slits close to Hensen's node, notochord, or in the lateral plate mesendoderm, and cell aggregates or beads were placed into the slit using insect needles.

Quail Embryo Model

The generation of the VAD quail model and the criteria for normal and VAD quail embryonic development *in ovo* have been described (Dersch and Zile, 1993). Briefly, vitamin A-adequate normal embryos were obtained from eggs of Japanese quail (*Coturnix coturnix japonica*) raised on the Michigan State University Poultry Research Farm and fed Purina Game Bird Chow. VAD quail embryos were obtained from eggs or quail fed a diet without vitamin A, but containing retinoic acid, which is not transferred to the egg (Dong and Zile, 1995). VAD embryos develop with high incidence of reversed cardiac situs (Zile *et al.*, 2000).

Statistical Analysis

All quantitative data were analyzed using Fischer's exact test. Statistical significance was set at $P < 0.05$.

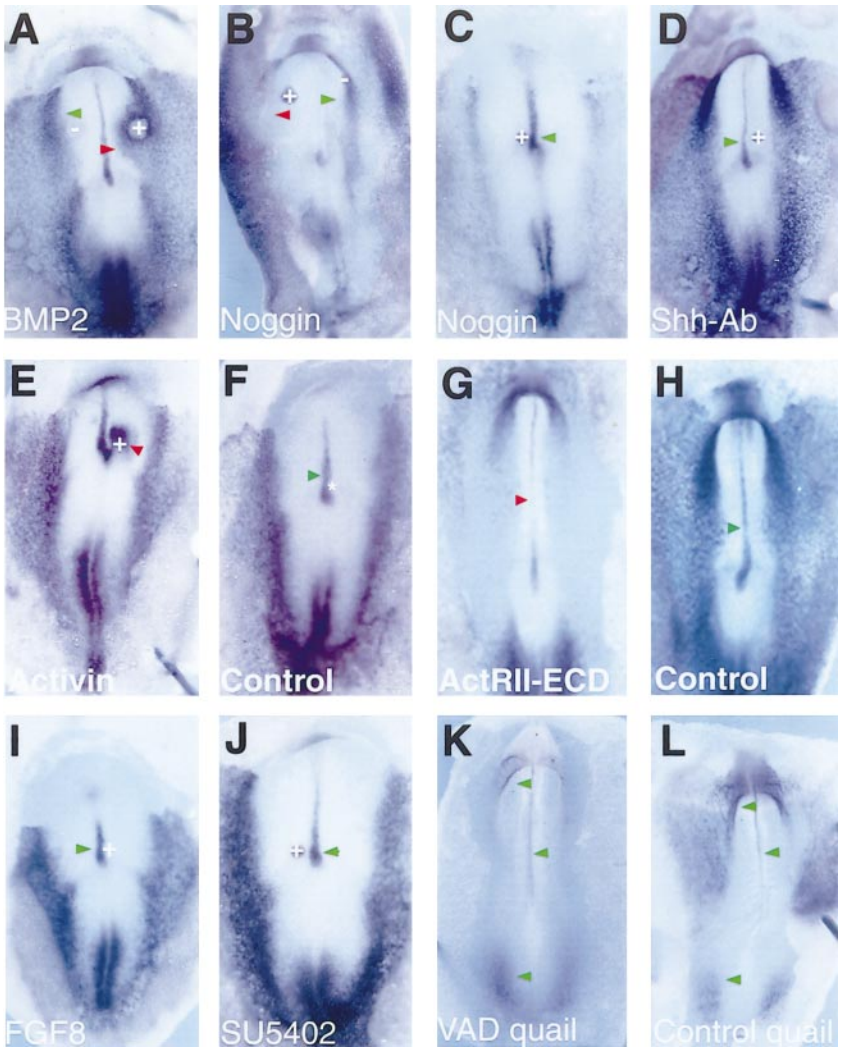


FIG. 2. Expression of chick CFC is dependent on BMP in the lateral plate and on an Activin-like signal in the embryonic midline. Expression of chick CFC was analyzed by whole-mount *in situ* hybridization of chicken embryos cultured *in vitro* between HH stages 4 and 7 (A–F, I, J), or between HH stages 6 and 8 (G, H). Chicken embryos were explanted and implanted with (A) aggregates of BMP2 expressing (+) and control cells (−). Cell aggregates were placed in the lateral plate mesoderm. (B, C). Aggregates of Noggin expressing (+) and control cells (−) were placed in the lateral plate mesoderm (B), or adjacent to Hensen's node (C). Beads loaded with (D) Shh antibody, (E) Activin A, (F) BSA, (I) FGF8, or (J) SU5402 were implanted on the left side of Hensen's node at HH stage 4. (G, H) Beads loaded with ActRII-ECD (G), or BSA as a control, were placed adjacent to the notochord of HH stage 6 embryos. (K, L) Chick CFC expression in (K) vitamin A-deficient (VAD) and (L) control quail embryos. Red arrowhead points to ectopic expression of chick CFC in (A) and (E), or loss of expression in (B) and (G). Normal expression domains are demarcated by a green arrowhead.

RESULTS

Identification of the Avian EGF-CFC Homolog, Chick CFC, Expressed in the Embryonic Midline, and Lateral Plate Mesoderm

To identify chick homologs of the EGF-CFC family, we performed a low-stringency screen, employing mouse Cfc2 cDNA as probe on a Hamburger–Hamilton (HH) stage 3–6 chick cDNA library. Several positive clones were isolated, sequenced, and revealed to be identical to a recently reported cDNA named chick-cripto (GenBank Accession No. AF282984; Colas and Schoenwolf, 2000). The mammalian Cryptic and Cripto genes have been recently renamed (Cfc1 and Cfc2, respectively; Bamford *et al.*, 2000). In order to comply with the new nomenclature, we propose to name this gene chick CFC.

Expression of the chick CFC gene was analyzed during early embryonic development by whole-mount *in situ* hy-

bridization. Chick CFC was expressed in the epiblast at Eyal-Giladi stage XI (Eyal-Giladi and Kochav, 1975) in an anteroposterior gradient with most intense expression around Köller's sickle (Figs. 1A and 1I). At HH stage 3, expression was seen in Hensen's node and in the primitive streak. The posterior half of the primitive streak was devoid of chick CFC transcripts. At HH stage 4, chick CFC was expressed in Hensen's node, the anterior half of the primitive streak, and in addition, in a halo surrounding Hensen's node (Fig. 1C). Between HH stage 5 and 7, expression in Hensen's node became asymmetric, being consistently stronger on the left side (Figs. 1D–1F, 1J). Symmetric expression domains were present in lateral plate mesoderm, the forming notochord, and in the prechordal plate (Figs. 1K and 1L). At HH stage 8, expression was found in cardiogenic mesoderm, posterior LPM, notochord, and in the posterior primitive streak (Figs. 1G and 1M). At HH stage 10, the myocardial layer of the tubular heart was prominently positive, while the endocardium was devoid of chick CFC expression (Figs. 1H and 1N).

Expression of Chick CFC Is Dependent on BMP in the Lateral Plate and on an Activin-like Signal in the Embryonic Midline

In order to identify signaling molecules which might control chick CFC expression in LPM and in the embryonic midline, we tested the effects of several candidate molecules. Implantation of cell aggregates expressing BMP2 adjacent to the LPM at HH stage 4 resulted in pronounced upregulation of chick CFC ($n = 26$; Fig. 2A). Conversely, implantation of cells expressing Noggin resulted in loss of chick CFC expression in LPM ($n = 17$), suggesting that BMP controls expression of chick CFC in LPM (Fig. 2B). In contrast, implantation of Noggin-expressing cells adjacent to the node or in the midline did not affect chick CFC expression ($n = 5$; Fig. 2C), suggesting that chick CFC is under a different control in the embryonic midline. Activin βB has been shown to repress Shh on the right side of Hensen's node at HH stage 4 and it might be responsible for the generation of asymmetric Shh expression in Hensen's node at HH stage 5 (Levin *et al.*, 1997). In order to test whether the asymmetric expression of chick CFC in Hensen's node might be the result of unilateral repression by Activin, beads loaded with recombinant Activin A were implanted on the left side of Hensen's node at HH stage 4. Surprisingly, Activin induced chicken chick CFC ectopically in paraxial mesoderm and enhanced the expression in the embryonic midline and in Hensen's node ($n = 9$; Figs. 2E and 2F). In order to interfere with Activin signaling, we used a soluble dominant negative Activin receptor (ActRIIA ECD; Ryan *et al.*, 1998). Beads coated with ActRII-ECD were implanted at HH stage 6 into or directly adjacent to the embryonic midline ($n = 12$). In the majority of implanted embryos (90%) a reduction or complete loss of chick CFC expression was observed, while embryos implanted with control beads ($n = 6$) revealed no alterations in chick CFC midline expression (Figs. 2G and 2H). Thus, it is reasonable to assume that an Activin-like signal might be the inducer of chick CFC in the embryonic midline, while asymmetric expression in the node cannot be the result of repression by Activin. We also tested the role of Shh by implanting beads loaded with neutralizing Shh antibody to the left side of Hensen's node. Shh antibody had no effect on chick CFC expression either in Hensen's node or in the notochord ($n = 13$; Fig. 2D). FGF8 has been recently identified as a right-sided signaling molecule in the chick that prevents the expression of left-sided signaling molecules such as *Car* on the right side (Boettger *et al.*, 1999). Neither beads loaded with recombinant FGF8 on the left side ($n = 15$) nor beads loaded with the FGF receptor antagonist SU5402 on the right side ($n = 12$) affected the asymmetric expression domain of chick CFC within Hensen's node or in the midline (Figs. 2I and 2J). Retinoic acid has been recently implicated in both mouse and chick as an important L–R determinant acting downstream of or parallel to Shh and is required for the proper expression of Nodal and Pitx2 in LPM (Chazaud *et al.*, 1999; Tsukui *et al.*, 1999; Zile *et al.*, 2000). In order to study the role of RA for chick CFC expression, we utilized the VAD model of total vitamin A deficiency in quail (Zile *et al.*, 2000). None of the expression domains in the LPM or notchord were affected by vitamin A deficiency (Figs. 2K and 2L).

Overexpression of Chick CFC, Mouse or Human CFC2 Results in Altered Heart Looping without Affecting the Expression of L–R Signaling Molecules

In order to test the effects of overexpressing chick CFC, mouse or human CFC2 in the chicken embryo, aggregates of cells expressing the different EGF-CFC peptides were implanted on the left or right side of Hensen's node at HH stage 4 and embryos were cultured overnight followed by analysis of cardiac looping morphogenesis. Alternatively, embryo development was terminated between HH stage 6 and 8 to analyze the expression pattern of different genes involved in L–R axis determination. Implantation of cells overexpressing human or mouse CFC2 on the left side directly adjacent to Hensen's node ($n = 7$), or more laterally either on the left ($n = 17$) or right side ($n = 10$), did not significantly affect heart looping (data not shown). However, implantation of cells expressing human or mouse Cfc2, secreted mouse Cfc2 lacking the hydrophobic C-terminus (Crl ΔC 25), or cells expressing chick CFC on the right side of Hensens node resulted in a statistically significant increased proportion of embryos with aberrant cardiac looping morphogenesis (Table 1; Fig. 3). Increased incidence of S-shaped hearts with a posterior ventricular segment looping to the left side, unlooped hearts, or left-looped hearts (L-loop) were observed with each cell implant (Table 1; Fig. 3). Several markers of L–R axis development including Nodal ($n = 8$), SnR ($n = 5$), Car ($n = 4$), and Pitx2 ($n = 32$) were studied by whole-mount *in situ* hybridization after implantation of human CFC2 and chicken CFC expressing cells. Despite the high incidence of abberant heart looping,

TABLE 1
Frequency of Embryos with Abberant Looping Morphogenesis after Right-Sided Overexpression of EGF-CFC Peptides

Implant	Total	Number (%) of embryos displaying			
		D-loop	S-shape	Unlooped	L-loop
Control (SiHA)	16	14 (93.3)	0	0	1 (6.7)
Control (COS-7)	14	13 (92.9)	0	1 (7.1)	0
Human CFC2[a]	27	16 (59.6)	2 (7.4)	4 (14.8)	5 (18.5)
Mouse Cfc2[b]	25	10 (40.0)	6 (24.0)	6 (24.0)	3 (12.0)
Mouse Cfc2ΔC 25[c]	14	9 (62.5)	1 (7.1)	2 (14.3)	2 (14.3)
Chick CFC[d]	16	10 (62.5)	2 (12.5)	3 (18.8)	1 (6.2)

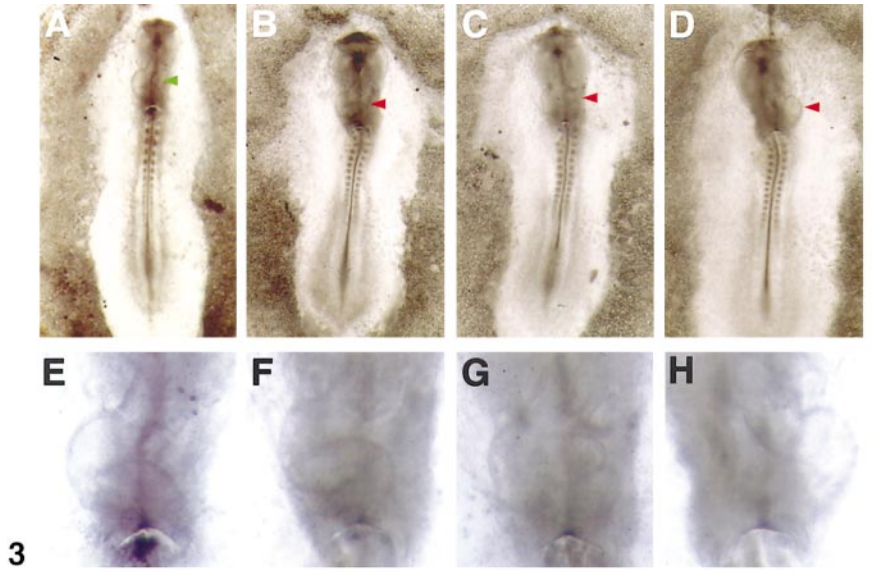
[a] Significant difference from control cells ($P < 0.003$).
[b] Significant difference from control cells ($P < 3 \times 10^{-5}$).
[c] Significant difference from control cells ($P < 0.03$).
[d] Significant difference from control cells ($P < 0.02$).

none of the studied genes showed any significant alteration in sidedness. Some variation in intensity of expression was seen between embryos; however, no correlation with the implanted cell type was noted (Fig. 4).

Chick CFC Controls Expression of Nodal in the LPM and Expression of Lefty1 in the Midline

In order to study the function of chick CFC further, several antisense oligodeoxynucleotides (ODN) targeted against different regions of the mRNA were designed. Two ODNs, either unmodified or phosphorothioate-modified, were found to be most effective in reducing chick CFC mRNA levels (Figs. 5E and 5J). Pluronic F-127 gel was used as a vehicle to apply ODNs regionally restricted (Makarenkova and Patel, 1999). Pluronic F-127 gel is liquid at low temperatures and forms a gel at room temperature, limiting diffusion from the region of application. Due to its properties as mild surfactant, the gel is thought to increase penetration of antisense ODN. In order to evaluate the distribution of applied ODN, FITC-labeled ODNs were applied to the surface of cultured embryos either centrally or laterally. The distribution of fluorescence suggested that the ODN-loaded Pluronic gel can be applied specifically to the embryonic midline or lateral plate (Figs. 5A and 5F). Fluorescence was no longer visible after 2 h of incubation (Figs. 5C and 5H); however, this does not necessarily reflect the time the ODN is biologically active. Chick CFC expression was strongly affected by antisense ODN treatment, suggesting efficient interference of gene expression by this experimental protocol (Figs. 5E and 5F).

Treatment of cultured embryos with two different antisense ODNs affected cardiac looping morphogenesis (Fig. 6; Table 2). Both ODNs induced a doubling of the number of embryos with abberant looping morphogenesis compared to control ODN treatment. However, only treatment with the phosphorothioate-modified ODN induced looping abberations which reached statistical significance. The presumably shorter half-life time of the unmodified oligonucleotide might affect its efficiency of inducing looping alterations. Both ODNs, however, proved to be efficient in altering L–R marker gene expression (see below). Whole-mount *in situ* hybridization was used to study the expression of various genes involved in L–R axis formation. Nodal expression was greatly reduced in the left LPM when antisense ODN was applied to the lateral plate (antisense ODN: 37.5%, $n = 24$ vs control ODN 7%, $n = 14$; $P < 0.05$; Figs. 7A and 7F). However, Nodal was expressed bilaterally both in LPM and in surrounding Hensen's node when antisense ODN were applied centrally (Figs. 7B and 7G; Table 3). Car was mostly found bilaterally expressed, but sometimes it was also absent (Figs. 7C and 7H; Table 3). Both homeobox genes, Pitx2 and NKX3.2, were ectopically expressed on the right side of the embryo. In addition, in one case NKX3.2 was present only on the right side and in another case was absent (Figs. 7D, 7E, 7I, and 7J). Bilateral expression of left-sided genes suggest that antisense ODN treatment resulted in left isomerization which may be explained by the deficiency of the midline barrier. *Lefty1* null embryos display left isomerism and bilateral expression of L–R determinants (Meno *et al.*, 1998). Therefore, Lefty1 expression was studied in antisense ODN-treated chicken embryos. Antisense ODN treatment resulted in loss of expression of Lefty1 when studied at HH stage 6 (antisense ODN: 73%, $n = 15$ vs control ODN: 13%, $n = 23$, $P < 0.05$; Figs. 8A and 8B). Interestingly, the loss of Lefty1 expression was only transient, as embryos stained for Nodal and Lefty1 at HH stage 8 revealed normal expression of Lefty1, while Nodal was bilaterally expressed (Fig. 8C). Thus, the observed bilateral expression of L–R marker genes is possibly the result of a transient loss of Lefty1 in the midline. Expression of SHH ($n = 10$) and FGF8 ($n = 12$) was not altered by the antisense ODN treatment (data not shown), suggesting a specific role of chick CFC in the control of Lefty1 expression.



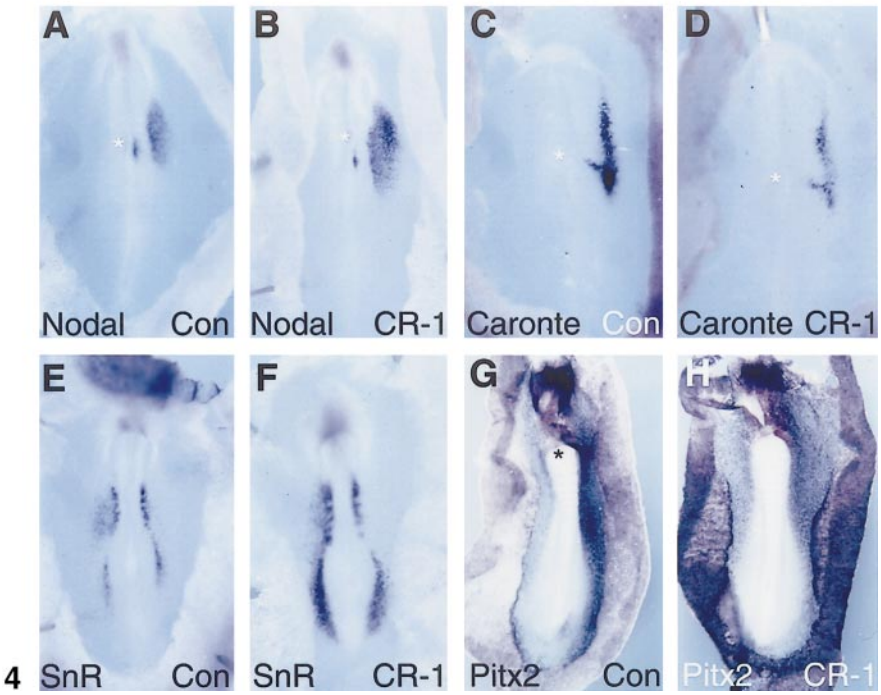
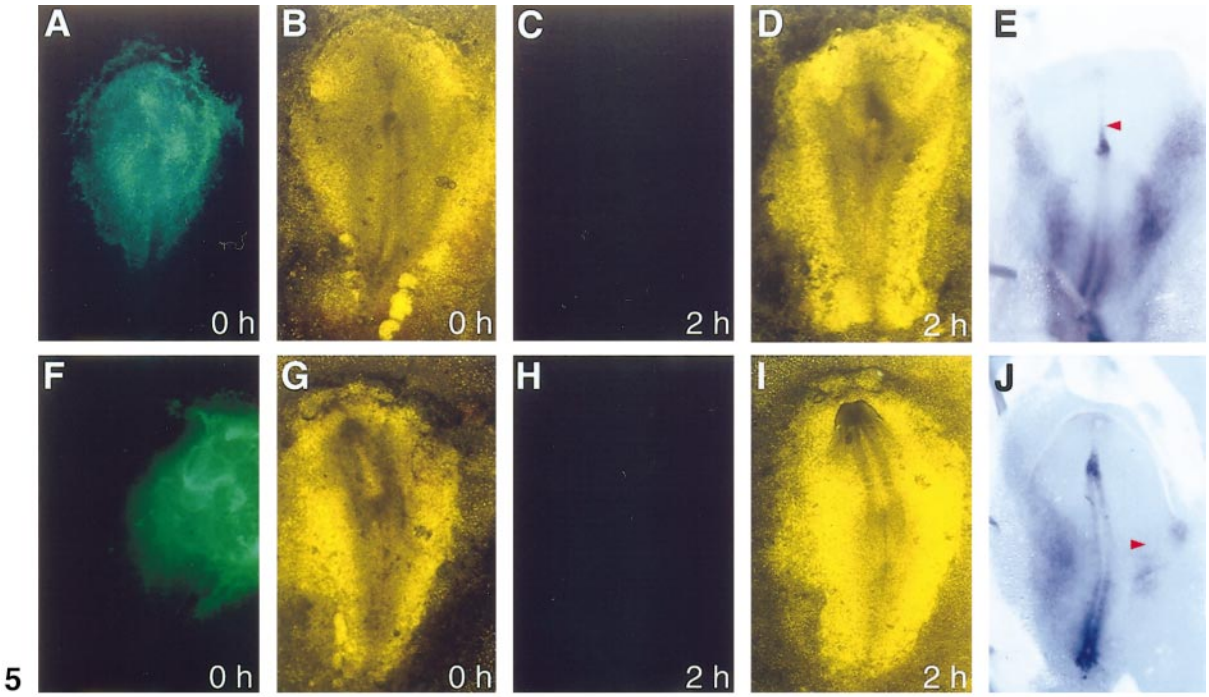


FIG. 3. Implantation of aggregates of SiHa cells expressing human CFC2 results in aberrant heart looping. Ventral views of (A) embryo with normal appearing D-loop; (B) embryo with S-shaped heart, arrow points to the caudal AV segment which loops to the left; (C) embryo with symmetric, unlooped heart; (D) embryo with leftward looping; (E–H) hearts of the embryos depicted in (A–D) shown at higher magnification.
FIG. 4. Overexpression of human CFC2 does not result in altered expression of L–R determinants. Ventral views of chicken embryos implanted with control (A, C, E, G) and CFC2 (B, D, F, H) overexpressing SiHa cells. Expression of (A, B) Nodal, (C, D) Car, (E, F) SnR, and (G, H) Pitx2 was examined by whole-mount *in situ* hybridization. Where possible the position of cell implants was labeled by an asterisk. Some variation in intensity of expression was seen between embryos; however, no correlation with the implanted cell type was noted.

DISCUSSION

By employing low-stringency hybridization, chick CFC was cloned and shown to be an avian member of the CFC-EGF family. The spatiotemporal expression of the chick CFC gene is very similar to the expression pattern of mouse Cfc1 (Shen *et al.*, 1997). It is presently unclear whether a CFC2-like cDNA exists in the chick. In the



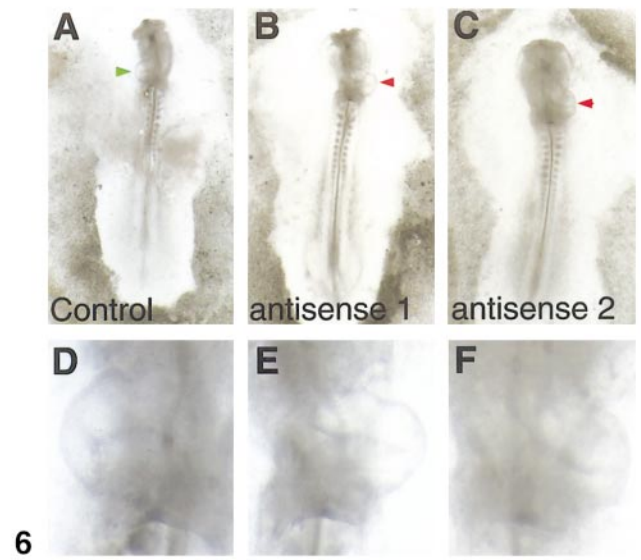


FIG. 5. Antisense ODN treatment results in localized downregulation of chick CFC expression. Pluronic gel containing FITC-labeled ODN were placed either around Hensen's node (A–E) or in the lateral plate (F–J) of cultured HH stage 4 embryos. (A, F) Localization of labeled ODN at the time of ODN application as indicated by the fluorescent signal. (C, D) After 2 h of incubation, a fluorescent signal is no longer detectable. (B, D, G, I) Phase-contrast images of the embryos shown in (A, C, F, H). (E, J) Embryos subjected to whole-mount *in situ* hybridization with a chick CFC probe after ODN treatment. Red arrowheads point to expression domains of chick CFC affected by ODN treatment.

FIG. 6. Central antisense ODN treatment of cultured chicken embryos results in altered looping morphogenesis. Ventral views of (A) embryo treated with control ODN with normal D-loop; (B, C) embryos treated with antisense ODN with L-loop. (D–F) Higher magnification of the cardiac region of embryos depicted in (A–C).

mouse, Cfc2 controls A/P patterning (Ding *et al.*, 1998), while Cfc1 is involved in L–R axis determination (Gaio *et al.*, 1999; Yan *et al.*, 1999). In zebrafish, both functions are mediated by the single gene oep (Shen and Schier, 2000). Despite the fact that we have employed a gastrula-stage cDNA library and a mouse Cfc2 cDNA probe for low-

TABLE 2

Frequency of Embryos with Abberant Looping Morphogenesis after Chick CFC Antisense ODN Treatment of the Embryonic Midline

		Number (%) of embryos displaying			
Treatment	Total	D-loop	S-shape	Unlooped	L-loop
Sense 2	45	38 (84.4)	0	4 (8.9)	3 (6.7)
Antisense 1[a]	56	38 (67.9)	4 (7.1)	7 (12.5)	7 (12.5)
Antisense 2[b]	64	46 (71.9)	2 (3.1)	8 (12.5)	8 (12.5)

[a] Significant difference from sense ODN treatment ($P < 0.04$).
[b] No significant difference from control cells ($P < 0.09$).

stringency screening, only a single cDNA species in the chick was found by us and independently by another group (Colas and Schoenwolf, 2000). Further experiments will address the question of whether chick CFC is also involved in A/P patterning and may mediate both CFC1 and CFC2 functions in the chick.

Expression of Chick CFC Is Controlled by BMP and Activin-like Signaling Pathways

During gastrulation, chick CFC was found to be expressed asymmetrically in Hensen's node and symmetri-

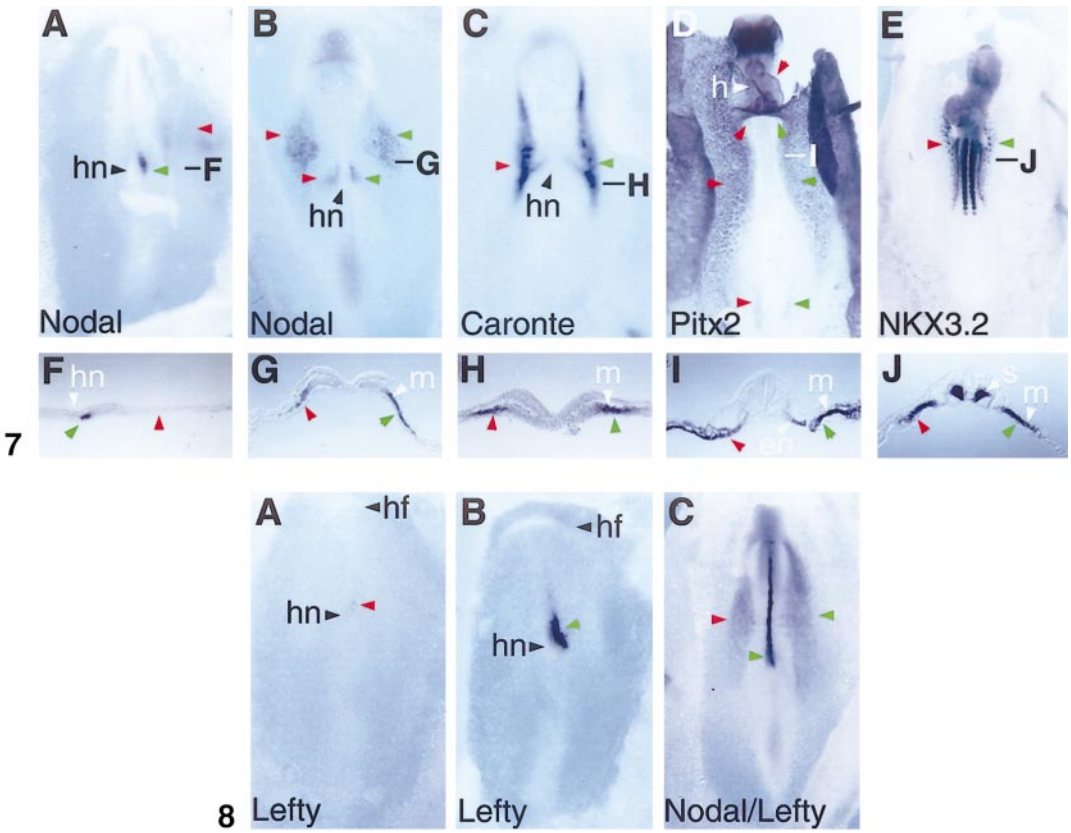


FIG. 7. Alteration of genes of L–R signaling pathway after antisense ODN treatment. *In situ* localization of (A, B) Nodal, (C) Car, (D) Pitx2, (E) NKX3.2 after (A) antisense ODN treatment of the lateral plate results in loss of Nodal expression in the LPM (red arrowhead), while Nodal expression in the left perinodal domain remains unaltered (green arrowhead). In contrast, antisense ODN treatment of the central region results in bilateral Nodal, Car, Pitx2, and NKX3.2 expression. Red arrowhead demarcates the ectopic right-sided expression domain, while the normal wild-type expression domain is marked by a green arrowhead. In (D) heart looping is reversed (red arrowhead). (F–J) Transversal sections through the embryos depicted in (A–E), respectively. Plane of sectioning is indicated in the individual panel. en, endoderm; h, heart; hn, Hensen's node; m, mesoderm; s, somite.

FIG. 8. Antisense ODN treatment for chick CFC results in transient loss of Lefty1. Ventral views of HH stage 6 (A, B) and HH stage 8 (C) embryos which were treated with antisense (A, C) or control (B) ODN in the central region at HH stage 4. Expression of Lefty1 (A, B) or a combination of Lefty1 and Nodal was examined after whole-mount *in situ* hybridization. (A) Antisense ODN treatment at HH stage 4 results in a complete loss of Lefty1 expression (red arrowhead) at HH stage 6, while in control ODN-treated embryos (B), a prominent expression domain in Hensen's node (green arrowhead) and in the forming notochord is visible. (C) The effect of the antisense ODN treatment is transient and at HH stage 8 a normal expression domain for Lefty1 was visible (green arrowhead) while Nodal was bilateral (red arrow demarcates the ectopic right-sided Nodal domain). hf, headfold; hn, Hensen's node.

TABLE 3
Chick CFC Antisense ODN Treatment of the Embryonic Midline Induces Bilateral Expression of L–R Determinants

			Expression (%)			
	Treatment	Total	Left sided	Bilateral	Right sided	None
Nodal	Control	26	26 (100.0)	—	—	—
	Antisense[a]	28	22 (78.6)	6 (21.4)	—	—
Car	Control	36	32 (88.9)	4 (11.1)	—	—
	Antisense[b]	31	20 (64.5)	8 (25.8)	—	3 (9.7)
Pitx2	Control	29	29 (100.0)	—	—	—
	Antisense[c]	85	58 (68.2)	27 (31.8)	—	—
NKX3.2	Control	7	7 (100.0)	—	—	—
	Antisense[d]	16	7 (43.8)	7 (43.8)	1 (6.2)	1 (6.2)

[a] Significant difference from sense ODN treatment ($P < 0.01$).
[b] Significant difference from control cells ($P < 0.02$).
[c] Significant difference from control cells ($P < 9 \times 10^{-5}$).
[d] Significant difference from control cells ($P < 0.01$).

cally in lateral plate mesoderm, notochord, and prechordal plate. A role for BMP signaling in the control of chick CFC expression in LPM was established by cell implantation. The result creates an apparent paradox. L–R signaling in the chick involves lateralization of Hensen's node, transport of L–R axis information to the lateral plate, and finally the execution of lateralization within organs. The BMP inhibitor Car transfers L–R axis information from Hensen's node to LPM. Car induces Nodal expression in LPM by locally inhibiting BMP signaling. In addition, genetic evidence suggests that oep in zebrafish and chick CFC in mouse are required for Nodal signaling in LPM (Gritsman *et al.*, 1999; Shen and Schier, 2000; Yan *et al.*, 1999). Thus, Nodal requires the inhibition of BMP signaling in order to be expressed in LPM but it also requires the presence of Cfc1 for signal transduction and maintenance of its own expression. As we have shown here, paradoxically, chick CFC expression requires the presence of BMP. BMP and Car might be temporally and spatially controlled in LPM and thereby sufficient BMP protein might be present to allow induction of chick CFC and also permits Nodal expression. A detailed high-resolution analysis of chick CFC, BMP2, and Nodal expression will be required to resolve this issue.

Chick CFC is asymmetrically expressed in Hensen's node. We therefore analyzed whether Noggin, Shh antibody, FGF8, SU 5402, or Activin A would affect the asymmetric expression domain. Only Activin A affected chick CFC expression in the midline, suggesting that an Activin-dependent pathway might control expression in the node and probably also in the notochord. Importantly, the dominant-negative receptor ActRII ECD blocked chick CFC expression in the notochord, complementing the gain of function experiments with Activin A. While Activin βA is not expressed in chicken embryos between stages 2 and 20 (Connolly *et al.*, 1995), Activin βB was found in Hensen's node and later in the notochord (Connolly *et al.*, 1995; Levin, 1998). Activin βB is therefore a potential candidate factor controlling chick CFC expression in the midline. Since chick CFC was also found on the left side of Hensen's node, where Activin βB is not present, another Activin-like signaling molecule that is expressed asymmetrically may control left-sided chick CFC expression in the node.

Overexpression of EGF-CFC Proteins Results in Aberrant Heart Looping Morphogenesis

Cardiac looping is a complex process involving at least three morphogenetic events including bending of the primitive ventricle to its ventral side, rightward rotation of the bending ventricle around a craniocaudal axis, and subsequent displacement of the conus to the right side (Männer, 2000). Overexpression experiments utilizing retrovirus expressing Nodal (Levin *et al.*, 1997), Pitx2 (Logan *et al.*, 1998), and Car (Yokouchi *et al.*, 1999), or loss-of-function experiments utilizing antisense ODN for SnR (Isaac *et al.*, 1997) resulted in altered cardiac looping morphogenesis. However, it was recently observed that sidedness of Pitx2 expression and heart looping did not strictly correlate, suggesting that looping morphogenesis may also involve Pitx2-independent events (Patel *et al.*, 1999). Consistent with this observation is the fact that Pitx2 null embryos display normal cardiac looping (Lu *et al.*, 1999). Both overexpression of EGF-CFC peptides and antisense ODN treatment for chick CFC reported here resulted in aberrant looping morphogenesis with no correlation to the sidedness of Pitx2 expression, supporting the fact that Pitx2 is not directly involved in cardiac looping. The existence of parallel pathways controlling cardiac looping is also suggested by the observation that inhibition of N-Cadherin function in Hensen's node results in aberrant looping morphogenesis and altered Pitx2 and SnR expression despite normal Nodal

and Lefty1 expression (Garcia-Castro *et al.*, 2000). Taken together, these results provide evidence that cardiac looping may be regulated by multiple signaling inputs, only some of which involve the established Nodal–Pitx2 pathway.

Overexpression of oep in zebrafish failed to reveal any significant phenotype which led to the suggestion that oep functions only as a permissive factor (Zhang *et al.*, 1998). However, in another study it was observed that overexpression of oep in both *Xenopus* and zebrafish embryos disrupted axis formation and affected Activin, Nodal, and BMP signaling, which would be suggestive of an instructive role for oep (Kiecker *et al.*, 2000). Moreover, in a recent study overexpression of human CFC1 in zebrafish embryos resulted in a gain-of-function phenotype including defective gastrulation (Bamford *et al.*, 2000). An active signaling function is also suggested by a large body of *in vitro* observations in epithelial cell lines after stimulation with human CFC2 (Salomon *et al.*, 1999). CFC2 elicits tyrosine phosphorylation of the ErbB4 receptor (Bianco *et al.*, 1999) and activates components of the Ras signaling pathway (Kannan *et al.*, 1997). Human CFC2 induces PI-3 kinase and increases phosphorylation of Akt and GSK-3β (Ebert *et al.*, 1999). Moreover, overexpression of CFC2 induces cell transformation (Ciardello *et al.*, 1991) and results in increased vimentin expression, an indicator for migratory epithelial cells (Ebert *et al.*, 2000). Interestingly, many of the effects seen with full-length CFC2 protein are also elicited using a peptide encompassing the EGF-like motif (Lohmeyer *et al.*, 1997). It is therefore formally possible that EGF-CFC family members might have two distinct functional domains, an EGF-like domain involved in the activation of the Ras and PI-3 kinase pathways and the CFC domain required for Nodal signaling. The observed high incidence of heart looping defects after implantation of cells overexpressing different EGF-CFC peptides in the absence of altered expression of genes involved in L–R signaling might be the result of activation of an EGF-like pathway. This hypothetical pathway might for example stimulate the migratory activity of ingressing mesodermal cells during gastrulation and thereby indirectly affect cardiac looping morphogenesis.

Nodal Expression in the Lateral Plate Mesoderm Is Dependent on Chick CFC

The use of Pluronic gel-mediated antisense ODN treatment gave us the opportunity to locally inactivate chick CFC gene expression and thereby define different functions of chick CFC in the embryonic midline and in the LPM. As demonstrated by antisense ODN treatment of the lateral plate, chick CFC is required for the maintenance of Nodal expression in the left LPM. Recently, strong evidence has been provided for a relationship between EGF-CFC activity and Nodal signaling (Gritsman *et al.*, 1999). EGF-CFC proteins appear to act as essential cofactors for Nodal signaling, based on the similarity of phenotypes between MZoep mutants and zebrafish Nodal mutants and on the requirement of oep activity for Nodal signaling. However, the Nodal/EGF-CFC interaction is not fully understood mechanistically and a direct interaction between Nodal and oep, or between oep and the putative Nodal type I receptor ActRIB, could not be demonstrated (Kiecker *et al.*, 2000). From *in vitro* and *in vivo* experiments it seems clear that Activin and Nodal utilize the same signaling pathway but Nodal requires in addition the presence of EGF/CFC peptides (Gritsman *et al.*, 1999; Saijoh *et al.*, 2000; Kumar *et al.*, 2001).

Lefty1 Expression in the Embryonic Midline Requires Chick CFC

Application of antisense ODNs to the embryonic midline resulted in bilateral expression of Caronte, Nodal, Pitx2,

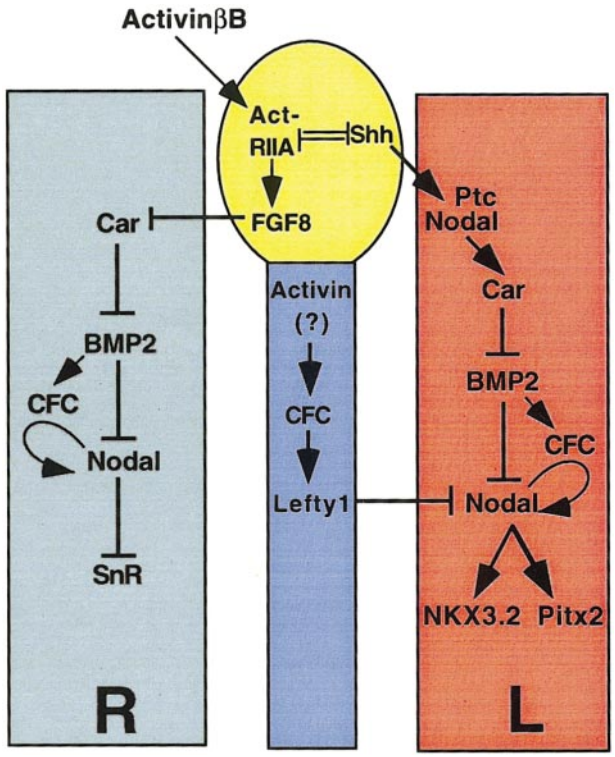


FIG. 9. Graph depicting the proposed role of chick CFC in L–R axis determination in the chick. Unilateral Shh and ActRIIA expression domains in the node (labeled yellow) are probably generated by a right-sided Activin βB signal at HH stage 3. Shh induces left-sided perinodal expression domains of Ptc, Nodal, and Car. Car inhibits BMP signal on the left side (labeled red) resulting in Nodal activation in LPM. Chick CFC is required for Nodal maintenance but not induction. BMP2 inhibits Nodal expression; however, chick CFC requires the presence of BMP2. Nodal expression results in the activation of NKX3.2 and Pitx2 in the left LPM. Chick CFC expression in the embryonic midline (labeled blue) is probably dependent on an Activin-like signal. Chick CFC is involved in the induction or maintenance of Lefty1 expression in the embryonic midline. While chick CFC is also expressed in the right LPM (labeled green), at present, no function can be assigned to this expression domain.

and NKX3.2. This result is in marked contrast to the data obtained in *Cfc1* null embryos and zebrafish late zygotic oep mutants (Gaio *et al.*, 1999; Yan *et al.*, 1999). In both mutants loss of Nodal, Lefty1, Lefty2, and Pitx2 expression was reported. We observed that in embryos treated with antisense ODN in the midline a transient loss of Lefty1 expression occurs. We therefore believe that the loss of chick CFC in the midline results in a phenocopy of the Lefty1 null mutant in mouse (Meno *et al.*, 1998). It has been proposed that Lefty1 functions as a midline barrier molecule preventing Nodal signaling from propagating across the midline. In both Lefty1 null mutants and chicken embryos treated with chick CFC antisense ODN, bilateral Nodal and Pitx2 expression is observed, resulting in left isomerization (Meno *et al.*, 1998). Experiments involving antisense ODN treatment of embryos *in ovo* will be required to study morphological effects of antisense ODN treatment on organ positioning and anatomy. The observed loss of left-sided gene expression observed in mutant mouse and zebrafish embryos is probably the result of the function of CFC1 and oep as a cofactors for Nodal signaling. Also antisense ODN in chick lateral plate treatment resulted in a loss of Nodal expression, suggesting that this function is evolutionary conserved. However, regionalized loss of chick CFC in the midline by antisense ODN treatment revealed an important additional role as a determinant of Lefty1 expression (Fig. 9). Future experiments will address the biochemical activity of chick CFC and its role during later stages of development.

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