FGF3 in the floor plate directs notochord convergent extension in the Ciona tadpole

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Convergent extension (CE) is the narrowing and lengthening of an embryonic field along a defined axis. It underlies a variety of complex morphogenetic movements, such as mesoderm elongation and neural tube closure in vertebrate embryos. Convergent extension relies on the same intracellular molecular machinery that directs planar cell polarity (PCP) in epithelial tissues, including non-canonical Wnt signaling components. However, it is not known what signals coordinate CE movements across cell fields. In the simple chordate Ciona intestinalis, the notochord plate consists of just 40 cells, which undergo mediolateral convergence (intercalation) to form a single cell row. Here we present evidence that a localized source of FGF3 in the developing nerve cord directs notochord intercalation through non-MAPK signaling. A dominant-negative form of the Ciona FGF receptor suppresses the formation of polarized actin-rich protrusions in notochord cells, resulting in defective notochord intercalation. Inhibition of Ciona FGF3 activity results in similar defects, even though it is expressed in an adjacent tissue: the floor plate of the nerve cord. In Xenopus mesoderm explants, inhibiting FGF signaling perturbs CE and disrupts membrane localization of Dishevelled (Dsh), a key regulator of PCP and CE. We propose that FGF signaling coordinates CE movements by regulating PCP pathway components such as Dsh.

KEY WORDS: Ciona, FGF, Ascidian, Convergent extension, Notochord, Planar cell polarity

INTRODUCTION

In vertebrates and ascidians, the formation of the primary embryonic body axis depends on the lengthening of the axial mesoderm by convergent extension (CE), a process in which cells intercalate medially along the anteroposterior (AP) axis (Keller, 2002; Wallingford et al., 2002). Members of the planar cell polarity (PCP) pathway, including Dishevelled (Dsh), Prickle and Van Gogh/Strabismus, are recruited to specific regions on the cell membrane upon activation of the non-canonical Wnt pathway (Lawrence et al., 2004; Jones and Chen, 2007; Seifert and Mlodzik, 2007) and transduce the Wnt/PCP signal into changes in actin dynamics and cellular motility. Disrupting these membrane protein complexes causes severe defects in tail extension and neural tube closure (Wallingford, 2006), suggesting a central role for these genes in chordate embryogenesis.

Tissue-level CE typically begins with coordinated polarization and movement of individual cells. During Xenopus mesoderm formation, cells across the dorsal marginal zone (DMZ) form actin protrusions and lamellodida preferentially along the mediolateral (ML) axis, events that are controlled by Dsh and other PCP genes (Wallingford et al., 2000). However, most of the known PCP components are intracellular molecules that function cell-autonomously to regulate cytoskeletal rearrangements. The putative extracellular signals that might establish the initial polarization of a field of cells have yet to be identified (Barrow, 2006; Casal et al., 2006; Lawrence et al., 2007).

Here, we present evidence for a non-autonomous mechanism for establishing ML polarity in the Ciona notochord. A localized source of FGF3 in the ventral midline of the neural tube signals to the underlying notochord plate to direct ML intercalation. Hence, FGF signaling could serve as the instructive signal for coordinating cell behavior over long distances and establishing the direction of CE in chordates.

MATERIALS AND METHODS

Ciona

Adult Ciona intestinalis were obtained and maintained as previously described (Corbo et al., 1997).

In situ hybridization and immunohistochemistry

In situ hybridization, diphosphorylated (dp) Erk staining and double antibody/in situ hybridization were performed according to Davidson et al. (Davidson et al., 2006). Phalloidin staining and lamellodida counting were performed according to Munro et al. (Munro et al., 2002a).

Morpholinos, PCR and enhancer constructs

C. intestinalis FGF3 (5'-CCGATGTTTGA-CTTACTTTGCGGCG-3') and splice acceptor (5'-CAATCTCA-GCTGTGAAAATAGAAAT-3') morpholinos were co-injected with Brachyury::GFP (10 ng/μl) at a total concentration of 1.5 mM. qRT-PCR was performed with total RNA extracted from ~15 embryos using SYBR chemistry on an ABI 3700 real time PCR system with the following primers:

- FGF3 (forward, 5'-CTTTACCTTTGGGGTGAGGT-3', reverse, 5'-GACATTCTGTCGGCGATTCCA-3')
- Cytoplasmic actin 7 (internal control, forward, 5'-GATTAGTCTGCTAGCTGCA-3', reverse, 5'-CATTTCCGGTGCCTGCTGCTGTTG-3')

A nerve cord-specific enhancer for the FGF3 homolog in Ciona savignyi (Cs-FGF3) was cloned by fusing the first intron (~6.2 kb) to a 3 kb genomic DNA fragment from the 5' flanking region. The nerve cord enhancer 00124 (5'-TATATAATCTTATCTTGTGCACAAC-3', 3'-TATTCTTTGTTAAACTGATTT-5'), notochord enhancer 00794 (5'-GCTGTGAAAATAGAAAT-3', 3'-TCGGTTCATAGC-5'), and endodermal strand enhancer 00794 (5'-CATCCTGAGAGATACA-3') were obtained and maintained as previously described (Corbo et al., 1997).

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For notochord cell protrusive activity, electroporated embryos were developed to the mid-neurula stage, dissociated in Ca²⁺/Mg²⁺-free medium with trypsin (Christiaen et al., 2008) and recovered for 15 minutes before mounting on a poly-l-lysine-coated slide. Movies were taken every 6 seconds over a period of 15 minutes.

**Xenopus explants**

Xenopus embryos were obtained, reared and injected as described (Sive et al., 2000). Embryos were injected in the animal hemisphere at the 2-cell stage for isolation of animal caps and equatorially into two dorsal blastomeres at the 4-cell stage for isolation of DMZ. Capped mRNAs were synthesized using the mMessage Machine Kit (Ambion) and injected as follows: Xdsh-GFP (Rothbacher et al., 2000) 120 pg; PKC-δ-YFP (Kinoshita et al., 2003) 100 pg; membrane RFP 200 pg. Animal caps (stage 9) were removed and cultured in 3/4× NAM (Sive et al., 2000) for 30 minutes before fixation and immunohistochemistry. The DMZ above the blastopore lip was excised from stage 10+ embryos in 1× Steinberg’s medium. DMZs were allowed to heal until whole-embryo siblings reached stage 10.5, then transferred to 1× Steinberg’s medium containing 120 μM SU5402 or DMSO and cultured at 15°C until stage 12. Embryos and explants were fixed in MEMFA (Sive et al., 2000), dehydrated in methanol, then rehydrated and processed for in situ hybridization or bleached in 1% hydrogen peroxide solution prior to immunohistochemistry. The following antibodies were used: chicken anti-GFP 1:500 (Abcam); rabbit anti-RFP 1:500 (Molecular Probes); Tor70 (notochord, mouse IgM) (Bolce et al., 1992); and 12/101 (muscle, mouse IgG) (Kintner and Brookes, 1984).

**RESULTS AND DISCUSSION**

Non-MAPK FGFR signaling is required for notochord convergent extension

In tadpoles of Ciona intestinalis, the notochord is composed of a single row of 40 cells that forms through ML intercalation, a common mode of CE (Miyamoto and Crowther, 1985). To identify potential regulators of notochord intercalation, we searched for genes that are specifically expressed in the notochord plate and found that the Ciona FGFR gene is strongly expressed in the notochord before and after intercalation (Fig. 1A,B). This expression is distinct from the role of FGFR signaling in notochord induction at the 32-cell stage of embryogenesis (Kim et al., 2000; Kim and Nishida, 2001), which depends on MAPK signaling through maternal FGFR and the transcriptional activation of Ciona Brachyury (Ci-Bra). By contrast, the later zygotic expression of FGFR in the notochord plate and definitive notochord was not associated with MAPK activity (Fig. 1G,H). Moreover, inhibition of MAPK activation with the pharmacological MEK inhibitor U0126 did not affect notochord intercalation when applied after the the notochord fate is specified (Fig. 1I).

FGFR signaling acts through four major downstream pathways: Ras/MAPK, PLCγ/Ca²⁺ and PI3K/Akt (Bottcher and Niehrs, 2005). To determine whether an alternative FGFR signaling pathway might be essential for notochord development, a dominant-negative form of the Ciona FGFR (dnFGFR) (Davidson et al., 2006) was specifically expressed in the notochord lineage after the late gastrula stage using the Ci-Noto1 enhancer (Takahashi et al., 1999). The transgene caused defects in notochord intercalation (Fig. 1C-F) but did not affect notochord cell fate specification (see Fig. S2 in the supplementary material). Many of the defective notochord cells failed to undergo appropriate shape changes (Fig. 1E’) and rearrangement. The resulting embryos had shorter and wider tails owing to the failure in CE (Fig. 1, compare F with D).

To better visualize notochord morphogenesis, we performed time-lapse microscopy on dorsal-anterior notochord/nerural tube explants (DA explants) (Munro and Odell, 2002b) isolated from wild-type and dnFGFR Ciona embryos. These DA explants preserve the normal spatial relationship between notochord and neural plate. In wild-type DA explants, notochord cells polarize, form dynamic membrane protrusions and intercalate along a distinct axis to produce an elongated structure (see Movie 1 in the supplementary material). By contrast, in DA explants from dnFGFR mutant embryos, notochord cells had diminished membrane protrusions and failed to intercalate, resulting in a wide notochord plate resembling that seen in wild-type embryos before CE (see Movie 2 in the supplementary material).

In Drosophila wing bristle development, Frizzled mutants exhibit both cell-autonomous and non-cell-autonomous PCP defects (Klein and Mlodzik, 2005). The Ciona FGFR seems to function strictly in a cell-autonomous manner. In mosaically transformed embryos, only those notochord cells that express the dnFGFR transgene displayed a rounded shape and failed to intercalate (Fig. 1E’). By contrast, neighboring wild-type notochord cells lacking the transgene underwent normal intercalation to form a single row of cells. Together, these data suggest that FGFR signaling – independent of the MAPK pathway – is essential for notochord intercalation in the Ciona tadpole.

**FGFR signaling controls polarized actin organization in notochord cells**

Lamellodid-like protrusions form at the ML edges of notochord precursor cells within the Ciona notochord plate (Munro and Odell, 2002a; Jiang et al., 2005) and are presumed to be the driving force for directional intercalation. To determine whether the dnFGFR transgene interferes with the formation of polarized lamellodida, phallloidin staining and confocal microscopy were used to reconstruct the three-dimensional organization of actin protrusions in the notochord (Fig. 1J). In wild-type embryos at the late neurula stage, an average of ~0.42 actin protrusions per internal edge was observed in the notochord plate, comparable with previous results from another ascidian species, Boltenia villosa (Munro and Odell, 2002a). Most of these protrusions were positioned within 45° of the ML axis as compared with the AP axis in the notochord plate, which is consistent with the ML direction of intercalation. When the dnFGFR transgene was expressed in the notochord, there was a ~50% reduction in the number of actin protrusions at internal notochord plate cell boundaries (Fig. 1J). Moreover, the remaining protrusions displayed a randomized orientation instead of the ~2:1 ML:AP ratio seen in wild-type notochord. This combined reduction in actin protrusions and polarity might underlie the defective notochord intercalation in dnFGFR embryos.

FGFR signaling is not only necessary for the formation of oriented actin protrusions in the notochord, but is also sufficient to induce ectopic cell protrusive activity. Dissociated wild-type notochord cells form random protrusions at low frequency in culture (Jiang et al., 2005) (see Movie 3 in the supplementary material). Incubating notochord cells with recombinant human basic FGF (bFGF) protein causes the cells to form multiple and larger protrusions at much higher frequency (see Movie 4 in the supplementary material), whereas cells expressing the dnFGFR transgene failed to respond to the ectopic FGF signal (see Fig. S5 and Movie 5 in the supplementary material). Thus, FGFR signaling might regulate Ciona notochord intercalation by inducing locally oriented, actin-based protrusions.

**Ciona FGFR3 is expressed in the floor plate above the converging notochord**

The preceding results suggest an instructive role for FGFR signaling in notochord intercalation. To identify the putative ligand, we examined the expression patterns of all six FGF genes in the Ciona genome.
FGF signal directs convergent extension

**Fig. 1. FGR is required for mediolateral convergence of the Ciona notochord.** (A,B) Ci-FGFR is strongly expressed in the developing notochord plate (A, neurula stage, white circle) and in notochord after intercalation (B, tailbud stage). (C-D') Wild-type notochord cells (Bra::GFP, green; phalloidin, red) undergo progressive ML intercalation (C, C', early tailbud) to form a single cell row (D, D', late tailbud). (E,F') Noto1::dnFGFR-Venus expressing (green) notochord cells display cell-autonomous defects in intercalation at early tailbud stage. (F,F') Noto1::dnFGFR+Bra::GFP (green) embryos display strong intercalation defects at late tailbud stage. (53/75 embryos displayed CE defects.) Images in C'-F' are magnifications from C-F, respectively. (G,H) dpErk staining of wild-type embryos at neurula (G) and late tailbud (H) stages shows absence of MAPK activation in the notochord. (I) Treatment of embryos with U0126 from late neurula stage on eliminates MAPK activation but does not affect notochord intercalation (103/103 embryos). (J) Distribution of the number of actin protrusions per internal notochord edge versus the ratio of ML- to AP-oriented protrusions. Note the difference between wild-type (blue) and dnFGFR (red) notochord indices (eight embryos each). P-values are indicated for each axis.

Only one of these, Ci-FGF3/7/10/22 (Ci-FGF3), is expressed in close proximity to the developing notochord (Imai et al., 2002). The Ciona dorsal nerve cord consists of four rows of ependymal cells in the tail (Crowther and Whittaker, 1992). Ci-FGF3 is expressed in the ventral-most row of cells, which constitutes the rudimentary floor plate of the nerve cord in tailbud stage embryos (Fig. 2A) and is located immediately above the midline of the notochord.

To determine the detailed expression pattern of Ci-FGF3, we characterized FGF3 regulatory DNA sequences. Because the Ci-FGF3 gene is located at the end of a contig in the C. intestinalis genome assembly, we used another ascidian species, Ciona savignyi. Previous studies have shown that orthologous enhancers work equally well in both species (Bertrand et al., 2003; Davidson et al., 2005). Indeed, an 11-kb Cs-FGF3 regulatory DNA fully recapitulated the normal FGF3 expression pattern in C. intestinalis when attached to a lacZ reporter gene, including staining in a single row of cells comprising the floor plate at tailbud stages (Fig. 2, compare C with A). At earlier stages, when the notochord plate starts to undergo intercalation, the Cs-FGF3 regulatory DNA drove reporter expression in the two rows of floor plate progenitors, which are in direct contact with the midline of the notochord plate (Fig. 2D,E). Later in development, the floor plate progenitors intercalate at the midline during neural closure to form the floor plate (Fig. 2F,G). Thus, it appears that Ci-FGF3 is expressed at the right time and place to serve as a signal for notochord intercalation.

**Ci-FGF3 is required for actin polarization and notochord convergent extension**

To address the function of Ci-FGF3 in Ciona notochord development, we designed two antisense morpholino oligos (MOs) targeting the splice donor/acceptor sites flanking the first intron of Ci-FGF3. qRT-PCR assays indicated that injection of both MOs caused a substantial reduction in the steady-state levels of Ci-FGF3 transcripts (see Fig. S3 in the supplementary material). Ci-FGF3 morphant embryos displayed a variety of CE defects, including noticeably shortened tails (Fig. 3A-C). Confocal imaging revealed several classes of mutant phenotypes with varying severity. In the most extreme cases, the mutant notochord consisted of two or three rows of cells instead of one, resulting in an extremely reduced tail that was about the same length as the trunk region (Fig. 3A-C'). To determine whether Ci-FGF3 regulates actin organization in the notochord, we performed phalloidin staining and confocal reconstruction of actin structures in Ci-FGF3 morphants. As seen for the dnFGFR transgene, the FGF3 MOs caused both a reduction in the number of actin protrusions and a randomization of protrusion orientation (Fig. 3E). The FGF3 morphant phenotype was stronger than that of dnFGFR mutants because the dominant-negative form of the receptor is not fully penetrant in the notochord.

Ci-FGF3 is expressed in a spatially localized pattern that corresponds to the ML polarity of actin protrusions. To determine whether FGF3 provides a positional cue triggering CE, we asked...
whether misexpressing FGF3 in neighboring tissues could affect notochord intercalation. We used four different tissue-specific enhancers to direct Ci-FGF3 expression in the nerve cord, notochord, tail muscles and endodermal strand. Such misexpression resulted in varying degrees of intercalation defects in the notochord (Fig. 3F-I’). The most severe defects were obtained upon misexpression of Ci-FGF3 in the notochord (Fig. 3F,F’), which emphasizes the need for the instructive cue to be expressed in a neighboring tissue. Relatively mild defects were obtained when Ci-FGF3 was misexpressed in the tail muscles (Fig. 3H,H’), which is consistent with the possibility that the muscles serve as a secondary source of intercalary signals, as seen in other ascidians (Munro and 

![Fig. 2. Ci-FGF3 is expressed in the ventral midline of the nerve cord during notochord CE. (A) Ci-FGF3 in situ showing expression in the floor plate at mid-tailbud stage. (B,C) Cs-FGF3 enhancer::lacZ in situ in late neurula (B; inset, high magnification) and mid-tailbud (C) embryos recapitulates endogenous Ci-FGF3 expression. (D,E) Cross-sectional (D) and lateral (E) views of late neurula embryos double labeled for Cs-FGF3 enhancer (green, lacZ in situ) and Bra::GFP (red, GFP antibody). (F) Cross-sectional view of late neurula embryo stained with phalloidin (green). The notochord plate (dashed line) and floor plate precursors (asterisks) are indicated. (G) Cross-sectional illustration of the organization of major Ciona tissues: ventral nerve cord (red), lateral nerve cord (light blue), dorsal nerve cord (purple), notochord (green), tail muscle (dark blue), endoderm (yellow) and epidermis (brown).

![Fig. 3. Ci-FGF3 is required for notochord CE. (A-C’) Phenotypic series of embryos co-injected with Ci-FGF3 splice morpholinos (MOs) and Bra::GFP (green) and counterstained with phalloidin (red). The notochord displays progressively stronger intercalation defects ranging from an occasional two cells per row (A,A’, category II), two-cell row (B,B’, category III) and more than two cells per row (C,C’, category IV). (D) Summary of MO phenotypes. n, number of embryos examined. (E) Distribution of actin protrusion abundance and ML/AP ratio in wild-type and MO embryos. The MO embryos display a more severe reduction in protrusions and loss of ML polarity than dnFGFR notochord (see Fig. 1J). (F-I’) Misexpression of Ci-FGF3 disrupts notochord intercalation non-cell-autonomously. Notochord (F,F’), ventral and dorsal nerve cord (G,G’), muscle (H,H’) and endodermal strand (I,I’) enhancer-driven Ci-FGF3 causes varying degrees of intercalation defects (number of defective embryos/total embryos) in the notochord. All embryos are co-electroporated with Bra::GFP (green) and counterstained with phalloidin (red). Images in A’-I’ are magnifications from A-I, respectively.]
supplementary material). Defects in notochord intercalary behavior did not result from changes in notochord fates (see Fig. S4 in the supplementary material).

FGFR signaling is required for membrane localization of Dishevelled and PKC-δ in Xenopus

One of the hallmarks of PCP and CE is the translocation of Dsh to the plasma membrane, which signals to regulators of actin dynamics to establish cell polarity (Habas et al., 2003). We asked whether FGFR signaling is required to promote membrane localization of the Dsh protein. This is hard to address in Ciona owing to the lack of Ci-Dsh antibodies and the variable penetrance of the dnFGFR transgene in the Ciona notochord. However, FGFR signaling has also been implicated in CE of the mesoderm in Xenopus laevis (Sivak et al., 2005). Therefore, we used Xenopus DMZ explants to assess the relationship between FGFR signaling and the subcellular localization of a Xenopus Dsh (Xdsh)-GFP fusion protein. As a control, we also assayed the localization of PKC-δ-YFP, which is a known downstream target of the FGFR pathway that becomes membrane-localized upon FGFR activation (Kinoshita et al., 2003; Sivak et al., 2005). Because FGFR signaling is required for specification of the mesoderm (including notochord) in Xenopus (see Fig. S6 in the supplementary material), we manipulated FGFR signaling by treatment with the FGFR pharmacological inhibitor SU5402 after mesoderm induction (from stage 10.5 onwards). This treatment results in short embryos owing to defects in CE (see Fig. S7 in the supplementary material).

In wild-type Xenopus DMZ explants undergoing CE, Xdsh-GFP and PKC-δ-YFP were localized primarily to the cell membrane (Fig. 4A,B). Upon SU5402 treatment, Xdsh-GFP was excluded from the membrane and was instead distributed throughout the cytoplasm (Fig. 4C), suggesting that FGFR signaling is required for Xdsh membrane localization. PKC-δ-YFP was exclusively localized to the cell membrane in wild-type DMZ explants, whereas the majority of the protein was present in the cytoplasm after treatment with SU5402 (Fig. 4D). As expected, DMZ explants did not elongate after drug treatment. Together, these data suggest that FGFR signaling is required for Dsh membrane localization in the Xenopus dorsal mesoderm during CE. Since Dsh is also required for CE in Ciona (Keys et al., 2002), similar mechanisms are likely to operate in Ciona notochord intercalation.

Directional movement of individual cells in embryogenesis can be achieved via diverse mechanisms, including chemotaxis, differential protrusive activity and differential adhesion. A key feature of CE movements is the coordination of uniform asymmetric cell behavior across large fields of cells. We have presented evidence that during Ciona notochord formation, FGF3 is released from the developing floor plate of the neural tube to coordinate ML polarity and CE movements of the notochord plate. This is consistent with the previous observation that ascidian DA explants can form elongated notochord rudiments in culture, whereas isolated notochord precursors alone do not (Munro and Odell, 2002b). Our results show that the instructive signal emanating from the neural plate to direct notochord CE in Ciona is a member of the FGF signaling pathway that works through the non-MAPK pathway downstream of FGFR. One candidate is the NRH receptor that functions downstream of FGFR to promote DMZ protrusive activity (Chung et al., 2005), although Ciona lacks a clear NRH homolog.

FGF signaling might play similar roles in coordinating complex morphogenetic processes in vertebrate development. As in Ciona, an early phase of MAPK-mediated FGF signaling is required to induce mesoderm fate in the frog embryo through the activation of genes such as Brachyury. After mesoderm specification, non-MAPK FGF signaling has been implicated in axial elongation in the Xenopus neurula (Sivak et al., 2005), but the details of this mechanism remain unclear. The complex and often overlapping expression patterns of 23 FGFs and four FGFRs in the early
Xenopus embryo (S.P., unpublished) prevent the unambiguous identification of a ligand-receptor relationship underlying CE, in contrast to Ciona. However, it is possible that a similar tissue-tissue interaction is involved in establishing the polarity of Xenopus DMZ cells.

Cells undergoing CE need to transduce the extrinsic polarity cues (such as FGFR) into coordinated (planar) cell behavior (Lawrence et al., 2002; Lawrence et al., 2004). It is likely that FGFR signaling achieves this effect through interacting with the non-canonical Wnt/PCP pathway. It has been shown previously that FGFR is required for membrane localization of PKC-δ, which physically interacts with Dsh (Kinoshita et al., 2003; Sivak et al., 2005). The Ciona genome encodes ten Wnts, but none of these is expressed in the notochord, raising the possibility they might play permissive roles in the notochord, as seen in other systems. We propose that a localized FGFR signal released by a developing tissue functions as an extracellular positional cue to directionally activate the intracellular PCP pathway in cells of an adjacent tissue (see Fig. S1 in the supplementary material). This coordination in cell polarity is the first step towards the orchestrated cell movements that underlie CE.

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Supplementary material
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