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Chemokine signaling controls endodermal migration during zebrafish gastrulation

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Abstract

Directed cell movements during gastrulation establish the germ layers of the vertebrate embryo and coordinate their contributions to different tissues and organs. Anterior migration of mesoderm and endoderm has largely been interpreted to result from epiboly and convergent-extension movements that drive body elongation. We show that the chemokine *cxcl12b* and its receptor *Cxcr4A* restrict anterior migration of endoderm during zebrafish gastrulation, thereby coordinating its movements with mesoderm. Depletion of either gene product causes the endoderm to separate from mesoderm and migrate farther anterior than normal, due to disruption of integrin-dependent cell adhesion. This results in bilateral duplications of endodermal organs, which may have relevance to human organ defects.

Keywords

Danio rerio; chemokine; gastrulation; gastrointestinal; alimentary

A crucial feature of vertebrate embryogenesis is the coordinated morphogenesis of germ layers (endoderm, mesoderm and ectoderm) during gastrulation (1). Interactions between endoderm and mesoderm specify organ locations and symmetries (2). Defects in endoderm alter morphogenesis of mesodermal organs (e.g. heart, kidneys, and blood), whereas mesodermal defects disrupt the locations of the liver and pancreas (2-5). Morphogenesis is regulated by Wnt (6) and Nodal signaling (7) when cells are intermingled in a bipotential “mesendoderm” (8). However, relatively little is known about germ layer-specific pathways that establish organ rudiments. In zebrafish, mesendodermal organ progenitors involute at the gastrula margin (blastopore) and move anteriorly toward the animal pole (future head) while converging toward the midline (convergent-extension).

The chemokine receptor CXCR4 controls directional migration in many contexts and is expressed in endoderm. It is upregulated by endodermal determinants, Mixer and Sox17 β (9-13) and is required for gastrointestinal vascularization (14). Of the two closely related zebrafish *Cxcr4s*, *Cxcr4B* regulates migration of many cell types (12,15-18), but no roles have been reported for *cxcr4A* during embryogenesis.

Zebrafish embryos deficient in *Cxcr4A* or *cxcl12b*, generated by injection with antisense morpholino oligonucleotides (MO), appeared morphologically normal (Fig. 1A-C; Fig. S1). However, analysis of *Tg(gutGFP)^{s854}* transgenic embryos in which the entire gut fluoresces (19) revealed duplications of endodermal organs at 56 hours post fertilization (hpf) (Fig. 1D-F; Fig. S2) including the pancreas (normally on the right; Fig. S3A-F) and liver (normally on the left; Fig. S3A-C, G-I), a phenotype we call “viscera bifida” (*cxcl12b*-MO: 78% n=23;

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cxcr4a-MO 68% n=37). At 26 hpf the intestine was also split bilaterally (Fig. 1G-I; *l2b*MO, 78% n=18, *4a*MO, 76% n=34), as revealed by *foxa2* expression, whereas the floorplate of the neural tube was unaffected (Fig. 1H, I). No defects were detected in mesoderm or ectoderm (Fig. S3J-O; Fig. S4).

During gastrulation, *cxcl12b* is expressed in mesoderm (Fig. 2A, C, E; Fig. S5A, B), whereas *cxcr4a* is expressed endoderm (Fig. 2B, D, F; Fig. S5E, F) and both require Nodal signaling (Fig. S5C, D, G, H), suggesting that chemokine signaling regulates endoderm-mesoderm interactions. At the onset of gastrulation (6 hpf), *sox32*+ endodermal cells appeared normal in number but were displaced slightly anteriorly in *cxcl12b* (83%, n=82) and *cxcr4a* (74%, n=62) morphants (Fig. 1J-L). Displacement became more pronounced by 8 hpf, as revealed by *foxa2* expression (Fig. 1M-O; *l2b*MO 88% n=34, *4a*MO 85% n=55). In controls, the trailing edge of *foxa2*+ cells was 50-100µm from the margin, whereas in morphants this gap was over 3-fold larger [178-275µm dorsally (D), 170-310 µm laterally (L), 186-340 µm ventrally (V)] (Fig. 1P). The leading edge was also displaced up to 100 µm, particularly ventrally. Displacement was not due to precocious endodermal internalization (Fig. S6), suggesting a later requirement for chemokine signaling in restricting endodermal movements anteriorly, toward the animal pole. We refer to this as endodermal “tethering”.

Despite displacement of the endoderm, mesoderm was unaffected as assayed by *no tail* (*ntl*) at 6 hpf (Fig. 1J-L), *tbx16* in paraxial mesoderm at 8 hpf (Fig. S4A-C), and *hand2* in lateral plate mesoderm (LPM, Fig. S4D-F). Our results reveal an early distinction between endodermal and mesodermal cell behaviors before they separate from mesendoderm and implicate the *cxcl12b*-*Cxcr4A* system as among the earliest known signals in endodermal morphogenesis.

If *cxcl12b* in mesoderm binds *Cxcr4A* in endodermal cells to restrict (tether) their movements, *Cxcr4A* should be required cell autonomously in the endoderm. To test this, we transplanted endoderm-targeted *cxcr4a* morphant cells into wild-type hosts (Fig. 2G-L). As an internal control, these cells (green-fluorescein) were co-transplanted with wild-type endoderm (red-rhodamine) into the same locations (Fig. 2G, I, K) in unlabeled hosts at 4 hpf, and cell distributions were compared 4 hours later (Fig. 2H, J, L). *cxcr4a* morphant endodermal cells moved, on average, 200µm further anteriorly than controls (Fig. 2M), demonstrating a cell autonomous requirement.

How does *cxcl12b*-*Cxcr4A* signaling regulate endodermal migration? Since *cxcl12b* is maternally deposited and localized to mesoderm, from which endodermal cells separate during gastrulation, it seems unlikely to act as a chemoattractant here. However, increasing evidence suggests that chemokine signaling modulates extracellular matrix (ECM) proteins [e.g. fibronectin (FN), laminin] and their receptors (integrins), which are required for gastrulation (20-24). FN in ECM binds secreted CXCL12 and presents it to CXCR4, causing its redistribution to leading edges of migrating cells (25). Of several *fns* in zebrafish, *fn1* is expressed by mesoderm during gastrulation (26). Thus, FN in the mesodermal ECM might bind and present *cxcl12b* to *cxcr4a*-expressing endoderm, sensitizing it to chemokines. CXCR4 activation by CXCL12 also enhances integrin-dependent adhesion of renal carcinoma and small-cell lung cancer cells to FN (22,27). Thus we considered both FN and integrins as potential downstream effectors during endodermal migration.

If this is correct, interfering with FN-integrin interactions should also disrupt endoderm migration. To test this, we treated gastrulating zebrafish embryos with RGD peptides (containing the tripeptide motif arginine-glycine-aspartate), which bind integrins and block signaling (28). This caused anterior displacement of endoderm at 8 hpf, similar to *cxcl12b* and *cxcr4a* morphants (Fig. 3A, B; 40%, n=52), and delayed convergence of endoderm towards

the midline (Fig. S7A-F; 55%, n=20). In contrast, convergence of LPM, which is required for gut morphogenesis (5), was unaffected (Fig. S7G, H) as in morphants (Fig. S4D-F). RGD peptide treatments of transgenic *Tg(gutGFP)^{s854}* embryos caused viscera bifida (Fig. 3C, D; 42% n=65) or situs inversus (Fig S7I, J; 14% n=64), even when applied at mid-late gastrula stages (viscera bifida, 32% n=22; situs inversus, 18% n=22), indicating that integrin-dependent interactions are essential throughout gastrulation.

These results argue against the presentation of FN-bound cxcl12b to Cxcr4A and instead suggest that chemokines control ECM-integrin-dependent adhesive interactions of endoderm. To test this, we assayed the ability of *cxcr4a* morphant endodermal cells to adhere to FN-coated surfaces in in-vitro cell adhesion assays. A third fewer morphant cells remained attached when compared to controls, and adhesion was rescued by co-injection of *integrin beta 1b* (*itgb1b*) mRNA (Fig. 3E), confirming that chemokine signaling directly regulates adhesion of endoderm to FN.

Molecular interactions between CXCL12 and CXCR4 upregulate levels of integrin α and β mRNAs in renal carcinoma cells to enhance their adhesion to FN (22). Thus, cxcl12b-Cxcr4A signaling in the endoderm may similarly regulate integrin levels. Of the integrin β s expressed in zebrafish, *itgb1b* is expressed maternally and ubiquitously during gastrulation (29). Quantitative real time-PCR revealed a reduction in *itgb1b* mRNA levels in whole embryos and in *cxcr4a*-MO endoderm (Fig. S7K), suggesting that the link between chemokines and integrins is, at least in part, a transcriptional one.

If endodermal defects in CXCL12b-CXCR4a morphants reflect disruption of ECM-integrin signaling, injecting *itgb1b* mRNA into morphants should rescue these defects. *itgb1b* mRNA injected into *cxcl12b* and *cxcr4a* morphants rescued intestinal bifurcations in a dose-dependent manner (Fig. 3F-K), either unilaterally (50-75 pg; Fig. 3I, J) or completely (100 pg; Fig. 3K; Table S1). Taken together, our results suggest that cxcl12b-Cxcr4A interactions promote integrin-mediated adhesion to tether endoderm to mesoderm during gastrulation.

Cell adhesion is a key regulator of gastrulation movements. E-cadherin mediates epiboly and anterior migration of prechordal mesoderm (30), integrin- α B allows mesendodermal cells to crawl on FN along the blastocoel roof (31), and *fn1* controls migration of myocardial progenitors towards the midline (26,32) - a process that also requires another chemokine, *apelin* (33,34). However, gut defects have generally been interpreted as secondary to defects in mesoderm migration. In contrast, our studies reveal an earlier requirement for ECM-integrin interactions directly in endodermal migration.

We show that endodermal migration towards the anterior is genetically separable from other gastrulation movements (35). We propose that chemokine-dependent expression of integrin tethers endoderm to mesoderm and that loss of this tether releases endoderm to move anteriorly (Fig. 4) secondarily causing viscera bifida because endodermal cells on either side, (presumably containing organ progenitors) (8), have farther to converge dorsally and do not reach the midline in time to fuse. Viscera bifida-like syndromes in humans, including intestinal cysts and ectopic pancreatic or liver tissue, are relatively common and not associated with spina bifida (ectoderm) (36), and defects in the CXCL12/CXCR4 signaling pathway may be an underlying cause.

For zebrafish endodermal cells, regulation of migration by controlling adhesion reconciles the recent observation that involuted endodermal cells initially move via a “random walk” rather than the directed migration displayed by mesoderm (37). Classically, chemokines are cytokines that induce chemotaxis in responding cells; CXCL12-CXCR4 interactions control homing of hematopoietic stem cells to the bone marrow, as well as migration of germ cells, neuronal progenitors and several metastatic cancers (15-18,27). In some of these cases, however, there

is evidence for a system more like the tether described here, where receptor-expressing cells are confined to a territory defined by ligand-expressing cells. Thus, chemokine-dependent changes in adhesion to the ECM may influence cell migration rates and directionality in many developmental and disease contexts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *cxcl12b* and *cxcr4a* are required for endodermal morphogenesis

A-C, Bright-field images, live embryos, 30 hpf. Controls (**A**) *cxcl12b* (**B**) and *cxcr4a* (**C**) morphants; **D-L**, dorsal views, anterior to the top. **D-F**, *Tg(gutGFP)^{s854}*, 56 hpf, reveals duplicated liver (L) and pancreas (P) (asterisks) in confocal projections of *cxcl12b* (**E**) and *cxcr4a* (**F**) morphants. **G-I**, *foxa2*, 26 hpf, the intestinal rod (ir) bifurcates but not floor plate (asterisks). **J-L**, Double in situ for *sox32* (endoderm-blue) and *ntl* (mesoderm-red), 6 hpf. *sox32* + cells, but not *ntl*+ or *sox32*+ forerunner cells (FRCs, arrowheads), move anteriorly (towards the animal pole and away from the margin). **M-O**, *foxa2*, 8 hpf, lateral views, morphant endoderm moves anteriorly (arrowheads indicate margin). **P**, quantitation of displacement of *foxa2*+ cells at dorsal (D), lateral (L, 90° from D) and ventral (V, 180° from D) positions, 8 hpf. Controls (red bars), *cxcl12b* morphants (blue bars) and *cxcr4a* morphants (gold bars). g: gut, hd: hepatic duct, pd: pancreatic duct, pe: pharyngeal endoderm.

Figure 2. Mesoderm expresses *cxcl12b* and endoderm requires *cxcr4a* cell-autonomously

A-F, Whole-mount in situ, dorsal right (except C, D - transverse sections of gastrulae). **(A)** *cxcl12b* in mesoderm, 8 hpf, arrow indicates margin, **(B)** *cxcr4a* in endoderm. **(C)** Double in situ for *cxcl12b* (blue) and *tbx16* (red), 8 hpf, confirms co-expression in region indicated by arrows, but not in endoderm (arrowhead). **(D)** Endodermal *cxcr4a* expression. **(E)** *cxcl12b* in lateral mesoderm (arrow), 10 hpf. **(F)** *cxcr4a* in endoderm and prechordal plate (PCP, arrow), 10 hpf. **G-L**, *cxcr4a* morphant (green) and control cells (red) grafted into unlabeled hosts, lateral views at 4.3-4.5 **(G, I, K)** and 8-8.5 hpf **(H, J, L)**. **M**, Average leading and trailing positions of control (red bars) and *cxcr4a* morphant (green bars) transplanted cells (n=7).

Figure 4. A chemokine-mediated tether model for endodermal morphogenesis

Diagrams of a wild type (WT) gastrula at 6, 8 and 10 hpf, and *cxcl12b/cxcr4a* morphants or RGD-treated (MO/RGD) embryos, lateral view, animal pole (top), dorsal (right). Endoderm (blue), mesoderm (red). Arrows: epiboly (1), involution (2), convergent-extension (3) and anterior migration (4). (A) At onset of gastrulation, *cxcl12b*⁺ mesoderm tethers *cxcr4a*⁺ endoderm which coordinates mesendoderm migration (B, C) By the end of gastrulation midline convergence clears ventral cells. (D) In morphants or RGD-treated embryos, endoderm released from this tether migrates anteriorly. (E, F) Displaced endoderm in anterior and ventral positions does not reach the midline (ectopic ventral blue dots in F), leading to organ duplications. Enlarged cells at center depict a molecular model in which *cxcl12b* from

mesoderm signals through Cxcr4A in endoderm to upregulate *itgb1b* expression, which binds FN1 in the mesodermal extracellular matrix.