Supporting Online Material for

Chemokine Signaling Controls Endodermal Migration During Zebrafish Gastrulation

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This PDF file includes:

Materials and Methods
Figs. S1 to S7
Table S1
References
SUPPLEMENTARY MATERIALS

MATERIALS AND METHODS

Zebrasfish: AB and Tg(gutGFP)^85d embryos were collected and staged under standard laboratory conditions.

Morpholino antisense oligonucleotides (MO): MOs (GeneTools) were designed against the start ATG for both cxcl12b (12bMO: 5`GCCTACTACTTTGCTATCCATGCC-3`) and cxcr4a (4aMO: 5`AGACGATGTGTTCGTAATAAGCCAT-3`) and were injected into 1-cell stage embryos at 10 ng for all experiments. The efficacy of each MO was tested by fusing the MO-target sequence upstream of eGFP. These constructs were injected either alone, or co-injected with the corresponding MO and knockdown of eGFP in MO-injected embryos was assayed in live embryos at 24 hpf (Fig. S1). A fluorescein-tagged 4aMO (4aMO-F), which gave identical phenotypes to the untagged 4aMO, was used for the endoderm-targeted mosaic experiments.

In situ hybridizations: Whole mount RNA in situ hybridizations were done as previously described (SI).

Endoderm-targeted mosaics: Donor AB embryos were co-injected with 500 pg of TARAM-A mRNA (S2) and 3% tetramethyl-rhodamine isothiocyanate (red). A second group of donors were co-injected with 500 pg of TARAM-A mRNA and 10 ng of 4aMO-F
Cells from both donors were co-transplanted into AB hosts at 4 hpf and imaged within 1 hour of transplantation and again at 8 hpf. Host embryos were raised to 24-30 hpf to confirm targeting of donor cells to the endoderm and only these embryos were taken into account for analysis.

**RGD peptide treatments:** RGD peptide (Sigma #G1269) was reconstituted to 4 mM in filter-sterilized embryo medium without methylene blue. AB or Tg(gutGFP)\textsuperscript{854} transgenic embryos were dechorionated and treated with 400 µl of the peptide at 1 mM in embryo medium at 8-cell or at 6 hpf and were fixed for foxa2 and hand2 in situs or confocal imaged at 56 hpf to visualize the gut.

**RNA rescue:** itgb1b was cloned into pCS2, linearized with NotI and sense mRNA synthesized using SP6 polymerase (mMessage mMachne kit, Ambion). AB, 12b and 4a morphants were injected with 50, 75 and 100 pg of itgb1b mRNA and rescue of foxa2 expression to a single intestinal rod was assayed at 30 hpf.

**Cell adhesion assay:** AB and cxcr4a morphant embryos were injected at the 1-cell stage with 500 pg of TARAM-4 mRNA and/or 100pg of itgb1b mRNA and raised to 4.3 hpf. 60-100 embryos were manually dechorionated in embryo medium with penicillin-streptomycin and dissociated into single cells (S3) in a 750 µl volume. Adhesion chambers were made by placing press-to-seal silicon insulators (20mm x 2.5 mm) onto plastic petridishes and coated with 10µg/ml fibronectin (Sigma) at 28°C for 4 hrs prior to use. The entire cell suspension was seeded into the chambers and incubated at 28°C for
2.5 hrs. Non-adhered cells were washed away by gently immersing the petridish in a beaker containing DMEM and placing it on a horizontal rotator for 5 mins. Average numbers of control and cxcr4a morphant cells that were stuck to the surface were counted by imaging 15 random, non-overlapping areas of each dish and averaged over 3 independent experiments.

**Quantitative RT-PCR:** AB embryos were injected with either cxcl12b or cxcr4a morpholinos (experiment 1) and also with 500 pg of TARAM-A mRNA (experiments 2 and 3). Total RNA was extracted using TRIZOL reagent and cDNA synthesized using Superscript III First-strand synthesis system for RT-PCR (Invitrogen). Quantitative RTPCR was set up using FastStart SYBR-Green Master mix (Roche) and primers 5’-GGCAGCAGATCTCAGGAAAC-3’ and 5’-TGGGAATGAGGTTTTTCAGC-3’ for itgb1b on an Opticon DNA Engine.
Figure S1: *cxcl12b* and *cxcr4a* morpholinos inhibit translation in vivo.

Live embryos, 30 hpf, lateral views. (A, C) GFP expression from injected mRNA in which either the *cxcl12b* (A) or *cxcr4a* (C) morpholino target sequence was fused upstream of GFP. (B, D) Sibling embryos co-injected with mRNA and *cxcl12bMO* (B) or *cxcr4aMO* (D) do not express GFP.
**Figure S2: Loss of chemokine signaling causes viscera bifida.**

Live embryos, 56 hpf, dorsal views, anterior to the top (A, E). Transverse serial sections of embryo in A, (B-D), and E, (F-H) stained with anti-GFP antibody. Horizontal dotted lines in A and E indicate the plane of sections in B-D and F-H, respectively. (A) In controls, the liver (L) and pancreas (P) lie on left and right sides of the gut (g), respectively. (B) The liver is located on the left side. (C) Posteriorly, the gut loops to the left and the pancreas is located to the right. (D) In a subsequent section, the gut forms a flattened tube at the midline. (E) The liver and pancreas are bilaterally duplicated in cxcr4a morphants. (F) Section through a cxcr4a morphant showing liver on both left and right sides and a narrow gut tube at the midline. (G) Section posterior to F showing similar bilateral pancreatic tissue. (H) Further posteriorly a single gut tube is present at the midline that resembles controls.
Figure S3: Defects in cexl12b and cexr4a morphants are restricted to endodermal organs.

Whole mount RNA in situ hybridizations, dorsal views, anterior to the top. (A-C) foxa3 expression, 30 hpf. In controls (A), foxa3 labels the liver (L) and pancreas (P). In morphants (B, 76% n=25; C, 48% n=21), foxa3 expression splits bilaterally. (D-F) insulin (ins) expression, 30 hpf. In controls (D), ins marks the pancreas at the midline, while in morphants (E, 72% n=74; F 67% n=86) ins+ are scattered bilaterally. (G-I) ceruloplasmin (cp) expression, 36 hpf. In controls (G), cp marks the liver on the left side, while in morphants cp expression is either bilaterally duplicated (H, 67% n=21; cexr4aMO, 59% n=22) or in some cases only on the right (I, 18% n=22; cexl12bMO, 19% n =21). (J-L) hand2 expression, 30 hpf. hand2 marks a single heart tube at the midline in controls (J), that appears unaffected in morphants (K, L). (M-O) dlx3b expression, 12 hpf. Expression at the border between neural and non-neural ectoderm (M) seems unaffected in morphants (N, O).
**Figure S4: Mesoderm progenitors are unaffected in cxcl12b and cxcr4a morphants.**

Whole mount RNA in situ hybridizations, dorsal views, anterior to the top. **A-C**, *tbx16*, 8 hpf, paraxial mesoderm (PM, arrowheads near margin) and prechordal plate (PCP, anterior midline arrowheads) mesoderm appears normal. **D-F**, *hand2*, 11hpf, lateral plate mesoderm (arrows) is unaffected.
Figure S5: *cxcl12b* and *cxcr4a* are expressed in nodal-dependant mesodermal and endodermal progenitors.

Whole mount in situ hybridizations. (A) *cxcl12b* in mesoderm, 6 hpf. (B) Mesodermal expression of *cxcl12b* (arrows) surrounding *cxcl12b*-negative endoderm (arrowheads), 8hpf. (C) 8 hpf, dorsal views, anterior to the top, *cxcl12b* expression in mesodermal progenitors, but not in the axial mesoderm (asterisk), resembles mesodermal markers.
such as tbx16 (see Fig. S4A-C) and is reduced in oep morphants (D). (E, F) cxcr4a in endoderm at 6hpf and 8 hpf respectively. (G) cxcr4a expression in endodermal progenitors includes dorsal forerunner cells (arrows) similar to other endodermal markers such as sox32 (see Fig. 1J-L). Similar to cxcl12b (D), cxcr4a (H) expression is reduced in oep morphants, in which the mesendoderm is severely reduced, confirming that both genes are expressed in mesendodermal cells.
**Figure S6: cxcl12b-Cxcr4A signaling does not act at the level of internalization of endodermal progenitors.**

Whole mount in situ hybridizations, 5.3 hpf, lateral views, anterior to the top. (A) *sox32* labels internalized endodermal progenitors at the margin immediately before gastrulation in control embryos. In *cxcl12b* (B) and *cxcr4a* (C) morphants internalized endodermal progenitors are similarly located at the margin.
Figure S7: RGD treatments phenocopy loss of chemokine signaling and chemokine morphants show reduced integrin expression.

A-J, whole mounts, anterior to the top. A, B, foxa2, 11 hpf, lateral views, dorsal - right. Some endodermal cells are delayed in dorsal convergence (to the right) in RGD-treated embryos. (C, D) foxa2, 11 hpf, dorsal views. In controls (C), foxa2+ endodermal cells from either side begin to coalesce into a single sheet across the midline, whereas in cxcr4a morphants (D, 63% n=19), these cells are spread more widely. (E, F) foxa2, 11
hpf, lateral views, dorsal - right. In controls (E), expression marks endodermal progenitors converging dorsally towards the midline. In RGD peptide treated embryos (F) convergence appears to be delayed in a subset of these cells. G, H, hand2 in lateral plate mesoderm, 11 hpf, dorsal views. Expression is unaffected in RGD-treated embryos (H). I, J) Confocal images of live Tg(gutGFP)\textsuperscript{s854} transgenic embryos, 56 hpf, dorsal views. In controls (I), the liver (L) and pancreas (P) lie on either side of the gut (g). In RGD peptide-treated embryos (J) organs are often inverted or duplicated. K, Quantitative RT-PCR shows downregulation of itgb1b in cxcl12b and cxcr4a morphant whole embryos (experiment #1, \( p = 0.002 \) and 0.04 respectively). itgb1b is also downregulated in cxcr4a morphant endoderm (experiment #2 and #3, \( p = 0.001 \) and 0.006 respectively).
Table S1: Quantitation of rescue of bilaterally split intestinal rod in chemokine morphants injected with itgb1b mRNA.

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SUPPLEMENTARY REFERENCES: