Chemokines are a large family of secreted proteins that play an important role in the migration of leukocytes during hematopoiesis and inflammation. Chemokines and their receptors are also widely distributed in the CNS. Although recent investigations are beginning to elucidate chemokine function within the CNS, relatively little is known about the CNS function of this important class of molecules. To better appreciate the CNS function of chemokines, the role of signaling by stromal cell-derived factor-1 (SDF-1) through its receptor, chemokine (CXC motif) receptor 4 (CXCR4), was analyzed in zebrafish embryos. The SDF-1/CXCR4 expression pattern suggested that SDF-1/CXCR4 signaling was important for guiding retinal ganglion cell axons within the retina to the optic stalk to exit the retina. Antisense knockdown of the ligand and/or receptor and a genetic CXCR4 mutation both induced retinal axons to follow aberrant pathways within the retina. Furthermore, retinal axons deviated from their normal pathway and extended to cells ectopically expressing SDF-1 within the retina. These data suggest that chemokine signaling is both necessary and sufficient for directing retinal growth cones within the retina.

Key words: zebrafish; retinal ganglion cell; SDF-1; CXCR4; axonogenesis; axon

Introduction
Chemokines are a large family of small, secreted proteins with important roles in the migration of leukocytes during inflammatory responses and in a variety of other processes, including development of the hematopoietic, lymphopoietic, and vascular systems (Rossi and Zlotnik, 2000; Tran and Miller, 2003) and migration by primordial germ cells (Doitsidou et al., 2002; Knaut et al., 2003) and lateral line sensory cells (David et al., 2002). A family of G-protein-coupled, seven-transmembrane receptors mediates chemokine action. Interestingly, chemokines and their receptors are expressed widely within the CNS (Cho and Miller, 2002) and despite their well-known role as chemotactic agents within the immune system, their function within the CNS is not well understood. Three known CNS targets of chemokines are the cells of the external granule layer in the cerebellum, granule cells in the dentate gyrus in the hippocampus, and retinal ganglion cells (RGCs). The chemokine, stromal cell-derived factor-1 (SDF-1), acts via the chemokine (CXC motif) receptor 4 (CXCR4) to mediate chemoattraction for migration by cerebellar and dentate granule cells. Interestingly, SDF-1/CXCR4 signaling can also regulate turning by cerebellar granule growth cones in vitro (Xiang et al., 2002), can reduce repulsion of RGC growth cones by Slit2 in vitro, and is required for proper pathfinding by the central axons of cutaneous sensory neurons (Chalasani et al., 2003a). These results suggest that SDF-1/CXCR4 signaling may mediate guidance of growth cones and perhaps other aspects of differentiation by neurons in vivo.

This report investigates the in vivo function of SDF-1/CXCR4 signaling for guidance of RGC axons in zebrafish embryos by using both gain- and loss-of-function strategies. Our results suggest that SDF-1/CXCR4 signaling plays an important role in the guidance of RGC growth cones to the exit site of the retina to the optic stalk.

Materials and Methods
Fish breeding and maintenance. Zebrafish were maintained as described previously (Westerfield, 1995), and embryos were staged by hour post-fertilization (hpf) (Kimmel et al., 1995). Wild-type, ath5:gfp (Masai et al., 2003), hsp70:gfp, and hsp70: sdf-1b-gfp were used in this study.

Cloning of zebrafish sdf-1b, sdf-1a, and cxcr4b. A 1.4 kb fragment of the sdf-1b cDNA was identified from a cDNA expression screen (C. Thisse and B. Thisse, unpublished observation). A full-length cDNA (2.9 kb) was cloned by using the fragment to screen a 32–36 hpf zebrafish Agtl1 cDNA library (gift from K. Zinn, California Institute of Technology, Pasadena, CA). Sequence analysis revealed that the cDNA encoded a zebrafish SDF-1 homolog and was named sdf-1b. A BLAST search using the sdf-1b sequence identified the zebrafish expressed sequence tag (EST) f88h12 (GenBank accession number AW420234) that encoded another full-length SDF-1 homolog that was named sdf-1a. A basic local alignment search tool (BLAST) search using the mouse CXCR4 sequence identified the zebrafish EST f10g03 (GenBank accession number
A1959485) that contained the full-length cxcr4b cDNA described previously (Chong et al., 2001).

Generation of expression constructs. Construct pHsp70/4-sdf-1b was made by inserting the sdf-1b cDNA into the pHsp 70/4-egfp vector (Holland et al., 2000) to replace the enhanced green fluorescence protein (EGFP) coding sequence. Construct pHis70/4-sdf-1b-egfp was made by amplifying the sdf-1b coding region from EST clone fj88h12 by PCR, cutting out the PCR product and inserting it into pEGFP immediately upstream to the egfp coding sequences. The coding sequence of SDF-1a-GFP fusion protein was then cut out and inserted into pHis70/4-egfp to replace egfp. Construct pHHsp70/4-cxcr4b-egfp was made by amplifying the cxcr4b coding sequence from EST clone fd10g03 by PCR, digesting the PCR product with EcoRI/BamHI, and cloning into pEGFP. The cxcr4b-egfp coding sequence was then cut out and inserted into pHis70/4-egfp to generate the construct.

Generation of hsp70/sdf-1b transgenic fish and induction of SDF-1b. Transgenic fish were generated as described previously (Xiao et al., 2003; Li et al., 2004), except that the presence of the transgene was assayed by PCR using primers from the Hsp70/4 promoter and the sdf-1b coding sequence. Three hsp70:sdf-1b-GFP lines (hsp70:sdf-1b-gfp1-3) and two hsp70:sdf-1b-3’utr-GFP lines (hsp70:sdf-1b-3’utr4-5) that were heat inducible were established. The three hsp70:sdf-1b-GFP lines varied in intensity of GFP fluorescence. For all experiments, hsp70:sdf-1b-GFP embryos that expressed the highest intensity of GFP fluorescence were used. Control hsp70:gfp embryos were generated previously (Holloran et al., 2000). Embryos were heat induced as described previously (Xiao et al., 2003).

In situ hybridization, immunocytochemistry, and retinal cryosection. In situ hybridization and antibody labeling to whole-mounted embryos was performed as described previously (Schulte-Merker et al., 1992; Westernfield, 1995). The following antibodies were used for immunocytochemistry: anti-GFP (1:25,000); monoclonal antibody (Mab) ZnS (1:500); anti-acetylated α-tubulin (1:1000). Ten micrometer transverse sections of the retina were prepared as described previously (Westernfield, 1995).

Morpholino knockdown. Morpholino oligonucleotides (MOs) with 0.1% phenol red were injected into 1- to 4-cell stage embryos. The following antisense MOs were used to inhibit the translation of sdf-1a or cxcr4b mRNA. The CAT sequence corresponds to the start ATG: antisense-sdf-1a MO, 5′-ACCTTGGATGCCATGTTGGCAGTG-3′; antisense-cxcr4b MO, 5′-AAATGATGTGGCATGTTAACATTCCC-3′.

In control experiments, standard randomized MO and a 4-base mismatched control MO for cxcr4b (5′-AAATGATGTGGCATGTTAACATTCCC-3′) were injected. Approximately 2 ng of the mixed antisense sdf-1a and cxcr4b MO (1 ng each) was injected into each embryo. The amount of MO injected was estimated by the volume of the injected solution as denoted by the phenol red.

Generation of transgenic/wild-type mosaic embryos and ectopic expression of SDF-1b-GFP. Cell transplantation from hsp70/sdf-1b-gfp to wild-type embryos was conducted as described previously (Ho and Kane, 1990). The micropipettes for transplantation were pulled using thin-walled glass tube (1 mm outer diameter) with a Sutter microelectrode puller. The tips were broken to 30–40 μm in diameter and then polished with a microforge. To better visualize transplanted cells in mosaic embryos, donor embryos were injected at 1- to 4-cell stage with fluorescent lineage tracer Alexa Fluor488 dextran (molecular weight, 10,000; Molecular Probes, Eugene, OR). Embryos were kept in embryo medium (Westernfield, 1995) at 28.5°C until the dome stage (4–5 hpf), and the chorions were removed manually. Embryos were mounted in a 3% methyl cellulose solution. The apparatus for transplantation was described by Westerfield (1995). Approximately 30–50 cells were injected into each host embryo near animal pole. After transplantation, mosaic embryos were raised in embryo medium and heat induced by placing embryos in a Petri dish into a water bath at 37°C at 28, 32, and 36 hpf for 0.5 h each and fixed at 40 hpf. Retinal axons were labeled with anti-acetylated α-tubulin, and donor cells were detected by GFP fluorescence or the fluorescent lineage tracer by confocal microscopy.

Results

Expression of SDF-1 and CXCR4 correlate with retinal axon outgrowth

In zebrafish, there are two cDNAs, sdf-1a and sdf-1b, that appear to be equally homologous to mammalian sdf-1. sdf-1b was identified from sequence and expression analysis of zebrafish cDNAs and sdf-1a from an EST identified from a BLAST search. The two cDNAs excluding the sequences encoding the signal peptides show 91% similarity with SDF-1a, 63% similarity with human SDF-1, and 64% similarity with SDF-1b. In zebrafish there are also two sdf-1 receptors, CXCR4a and CXCR4b, that are highly homologous to each other and equally homologous to mammalian CXCR4, suggesting that the CXCR4 gene was duplicated in the zebrafish genome (Chong et al., 2001). The high level of homology between sdf-1a and sdf-1b and the equal homology between them and human SDF-1 suggests that sdf-1a and sdf-1b are duplicates much like many other genes in teleost genomes (Postlethwait et al., 2000).

The two sdf-1 genes are expressed in specific and dynamic patterns with little overlap (Li et al., 2004). One place where both sdf-1s are expressed is by cells in the portion of the optic stalk that is adjacent to the eye (Fig. 1A, B). In the optic stalk, sdf-1a expression extends right up to the retina, whereas sdf-1b is expressed more distally from the retina. Embryos hybridized with an sdf-1a riboprobe and their retinal ganglion cell axons labeled with anti-acetylated α-tubulin showed that the axons extend along cells expressing sdf-1a (data not shown). Furthermore, cxcr4b but not cxcr4a is expressed by cells that, by their position, are likely to be retinal ganglion cells during the period of their axonogenesis (Fig. 1C) (Chong et al., 2001). Thus, the expression of the two chemokines and their putative receptor suggest that SDF-1 secreted from the optic stalk attracts the axons of CXCR4 bearing RGCs to the exit site of the retina into the optic stalk.

Antisense knockdown of SDF1/CXCR4 signaling leads to pathfinding errors by retinal ganglion axons

Because the sdf-1s are well situated to guide RGC axons to the exit site of the retina, we wondered whether interference with SDF-1/CXCR4 signaling might cause RGC axons to follow aberrant pathways within the retina. To determine whether SDF-1/CXCR4 signaling is required for guidance of RGC axons, we in-
Figure 2. MO against sdf-1a efficiently and specifically knocks down the synthesis of SDF-1a. All panels show 28 hpf embryos that were heat induced at 24 hpf. A, Wild-type embryo co-injected with hsp70-sdf-1a-gfp expression construct and standard control morpholino, showing mosaic expression of SDF-1a GFP after heat induction. B, Co-injection of hsp70-sdf-1a-gfp plasmid and antisense sdf-1a-MO effectively blocked protein synthesis of targeted transcript, as indicated by the absence or severe reduction of GFP fluorescence. Because the heat-inducible expression of hsp70-sdf-1a-gfp construct is much higher than the endogenous expression of sdf-1a, similar dosage of antisense sdf-1a-MO can presumably effectively knock down endogenous sdf-1a expression as well. C, A heat-induced hsp70:sdf-1b-gfp transgenic embryo injected with antisense sdf-1a morpholino, showing induced, ubiquitous expression of SDF-1b GFP, of which expression was not affected by antisense sdf-1a-MO. Scale bar, 40 μm.

Figure 3. Knockdown of SDF-1a and CXCR4b led to pathfinding errors by RGC axons. All panels are ventral views of retinas of 48 hpf ath5:gfp transgenic embryos. A, The optic nerve and RGCs marked by GFP fluorescence in a 48 hpf transgenic embryo injected with antisense sdf-1a morpholino, showing mosaic expression of SDF-1a GFP after heat induction. B, Coinjection of hsp70-sdf-1a-gfp plasmid and antisense sdf-1a-MO effectively blocked protein synthesis of targeted transcript, as indicated by the absence or severe reduction of GFP fluorescence. Because the heat-inducible expression of hsp70-sdf-1a-gfp construct is much higher than the endogenous expression of sdf-1a, similar dosage of antisense sdf-1a-MO can presumably effectively knock down endogenous sdf-1a expression as well. C, A heat-induced hsp70:sdf-1b-gfp transgenic embryo injected with antisense sdf-1a morpholino, showing induced, ubiquitous expression of SDF-1b GFP, of which expression was not affected by antisense sdf-1a-MO. Scale bar, 40 μm.
Pathfinding by retinal axons is disrupted in cxcr4b mutant odysseus embryos

The odysseus (ody) mutant was first identified by a defect in migration by primordial germ cells caused by a putative loss-of-function mutation in the cxcr4b gene (Knaut et al., 2003). Because knockdown of SDF-1/CXCR4 signaling caused RGC axons to follow aberrant pathways in the retina, we examined axonal outgrowth by RGCs in odysseus embryos. Indeed, retinal axons projected in erroneous directions to form prominent tracts within the retina of all eight mutants examined (Fig. 6). In many cases, axons extended away from the exit site of the retina. Interestingly, in some cases, projection errors were only seen in one of the two eyes in mutants much like that observed in SDF-1a/CXCR4b morphants (Fig. 4). As in the case of embryos with SDF-1/CXCR4 signaling knocked down, observation of the retina in mutants with differential interference contrast microscopy revealed no obvious defects (data not shown). This result strongly supports the knockdown result and demonstrates that SDF-1/CXCR4 signaling is important for correct pathfinding by RGC axons to the exit point of the retina. Furthermore, because RGCs project axons in both sdf-1a/cxcr4b antisense morphant embryos and in odysseus embryos, SDF-1/CXCR4 signaling appears not to be required for outgrowth of RGC axons but rather serves a pathfinding function for these axons.

Retinal axons are attracted by retinal cells misexpressing sdf-1

Errors by RGC axons after knockdown of SDF-1/CXCR4 signaling in wild-type embryos and in odysseus mutants that signaling through this chemokine serves a pathfinding function but does not explain how SDF-1 guides RGC axons. The expression pattern of sdf-1a and sdf-1b by the optic stalk suggests that SDF-1 attracts RGC axons to the optic stalk. To test this hypothesis, pathfinding by RGC axons was examined in retinas that contained ectopic sources of SDF-1 to determine whether the axons were attracted by the ectopic sources. This was accomplished by transplanting cells from hsp70-sdf-1b-gfp transgenic embryos into wild-type hosts at 4–5 hpf and heat-inducing expression of the SDF-1b GFP at 28, 32, and 36 hpf and assayed for RGC axons with anti-acetylated α-tubulin at 40 hpf. Under these conditions, embryos were mosaic with transgenic cells scattered randomly within the wild-type host. Control embryos were
transplanted with cells from hsp70:gfpk6 embryos and heat induced. In control embryos, the pathways of RGC axons were normal in all retinas (n = 33) that contained GFP-expressing cells. However, RGC axons were found in aberrant positions along or within 2 μm from SDF-1b GFP-expressing cells within the retina in 40% of retinas that contained GFP-expressing cells (n = 25) in experimental embryos (Fig. 7). The correlation of deviant RGC axons and SDF-1b GFP-expressing cells suggests that SDF-1 acts as a chemoattractant for RGC axons and that SDF-1b and perhaps SDF-1a secreted by the initial portion of the optic stalk serves to attract RGC axons to the head of the optic nerve to guide them out of the retina.

**Discussion**

**Axonal guidance by SDF-1/CXCR4 signaling**

Both knockdown of SDF-1/CXCR4 signaling and mutations in cxcr4b adversely affected the pathways followed by RGC axons. Furthermore, the pathways followed by RGC axons were aberrant in retinas ectopically expressing SDF-1, and the pathways were along or in the vicinity of ectopically expressing cells. The simplest interpretation of our results is that SDF-1s may serve as a chemoattractant for RGC growth cones. This interpretation can account for the strikingly similar retinal axon phenotypes observed in shh mutants and knock-outs (Schauerte et al., 1998; Dakubo et al., 2003). In these embryos, a loss of Shh from the retinal ganglion cells leads to defects in the differentiation of the optic stalk, which could lead to a loss of stalk-derived SDF-1 and subsequent retinal axon defects. The idea that SDF-1 is a chemoattractant for retinal axons is supported by the fact that SDF-1 is a chemoattractant for cells in the immune system and migrating cerebellar and dentate granule cells (Cho and Miller, 2002). Consonant with this idea, other molecules such as semaphorins, netrins, slits, and ephrins regulate both cell migration and growth cone guidance (Park et al., 2002). Furthermore, SDF-1/CXCR4 signaling can affect turning by growth cones in vitro and path-finding by cutaneous sensory axons in the spinal cord (Xiang et al., 2002; Chalasani et al., 2003a). Counter to this hypothesis, in vitro SDF-1 apparently exerts no direct chemoattractive effect on
chick RGC axons (Chalasani et al., 2003a). This might be because of species differences and/or in vitro versus in vivo differences or differences in the context or state of the growth cones. In fact, SDF-1α has been reported to both induce apoptosis and promote survival or proliferation of neurons (Cho and Miller, 2002), suggesting that context may influence chemokine action. Finally, the response of growth cones to guidance molecules such as Netrin and Semaphorins can vary depending on the status of intracellular messengers Ca2+, cAMP, and cGMP (Song and Poo, 1999). Thus, the action of SDF-1 can vary, depending on the context, and could potentially explain the disparity in the in vitro and in vivo results.

A second possible mechanism for SDF-1/CXCR4 effects on pathfinding is the regulation of the action of other guidance molecules by SDF-1/CXCR4. For example, SDF-1/CXCR4 may potentiate the effects of Netrin/deleted in colorectal cancer (DCC). Netrin-1 is expressed by the head of the optic stalk in a similar manner to the SDF-1s and can stimulate neurite outgrowth from RGCs (Deiner et al., 1997; Lauderdale et al., 1997). Furthermore, in Netrin-1 and DCC knock-outs, RGC axons extend normally to the optic disc but failed to exit the eye and instead extended aberrantly within the eye. This suggests that Netrin-1/DCC signaling is not necessary for guidance to the optic disc and thus does not mediate an essential chemoattractive function but is required for exiting the eye. This contrasts with RGC axons in SDF-1a/CXCR4b knock-down embryos that failed to extend to the optic stalk and instead extended aberrantly within the eye. Because the Netrin/DCC and SDF-1/CXCR4 loss-of-function phenotypes differ from each other, it is unlikely that SDF-1/CXCR4 simply acts to potentiate the action of Netrin/DCC on RGC axons.

SDF-1/CXCR4 can interact with various axonal repellants, and this could potentially account for the pathfinding errors made by RGC axons in our experiments. Slit-1 and Slit-2 inhibit RGC axons (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000; Plump et al., 2002), and application of SDF-1 in vitro reduces the repulsive action of Slit-2 (Chalasani et al., 2003a). Slit-1a and Slit-1b are both expressed in the zebrafish retina and Slit-3 by the lens, but Slit-2 is not expressed in the retina (Yeo et al., 2001; Hutson and Chien, 2002; Hutson et al., 2003). Thus, silencing of repulsive actions of Slit-1a and/or Slit-3 by SDF-1/CXCR4 could participate in guiding RGC axons within the zebrafish retina. SDF-1/CXCR4-mediated silencing of repulsive molecules may also play a role in guidance of RGC axons within the optic stalk and chiasm. Slit-2 is expressed by the optic stalk in both mammals and zebrafish (Nicolou et al., 2000; Hutson and Chien, 2002), and RGC axons make errors in and near the optic chiasm in double knockout mutants of Slit-1 and Slit-2 (Plump et al., 2002) and in zebrafish mutant for robo2 that encodes a Slit receptor (Fricke et al., 2001). Thus, previous exposure of RGC growth cones to SDF-1s in the optic stalk could potentially reduce the repulsive action of Slit-2 to allow RGC axons to extend through the optic stalk and chiasm.

SDF-1, Netrin-1, and Slit-2 all affect RGC axon extension. How might these molecules act within the visual pathway to guide RGC axons? The SDF-1s and Netrin-1a are expressed by the head of the optic stalk in zebrafish. SDF-1 proteins are small proteins that may diffuse farther than the larger Netrin-1, which is thought to act locally at the optic disc (Deiner et al., 1997). We propose that the SDF-1s are distributed as a gradient within the retina and act as a chemotactant to guide RGC axons within the retina to the optic stalk. Once at the head of the optic stalk, the growth cones interact with Netrin-1, which guides them out of the eye into the optic stalk. Furthermore, within the optic stalk and chiasm, previous exposure of RGC growth cones to SDF-1s in the head of the optic stalk then reduces the repulsive effects of Slit-2 to allow them to traverse through the stalk and chiasm. Finally, SDF-1/CXCR4 signaling also mediates a trophic activity on RGCs (Chalasani et al., 2003b). Thus, SDF-1/CXCR4 signaling may be active both for trophic support of neurons and guidance of their axons.

References


