

The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival

Kathleen A. Molyneaux^{1,*}, Hélène Zinszner^{2,*}, Prabhat S. Kunwar², Kyle Schaible¹, Jürg Stebler³, Mary Jean Sunshine⁴, William O'Brien⁴, Erez Raz³, Dan Littman⁴, Chris Wylie^{1,†} and Ruth Lehmann^{2,†}

¹Division of Developmental Biology, Children's Hospital Research Foundation, Cincinnati, OH 45229, USA

²Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, New York University Medical Center and Howard Hughes Medical Institute, New York, NY 10016, USA

³Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Gottingen, Germany

⁴Molecular Pathogenesis Program, Skirball Institute of Biomolecular Medicine, New York University Medical Center and Howard Hughes Medical Institute, New York, NY 10016, USA

*These authors contributed equally to this work

†Authors for correspondence (e-mail: Lehmann@saturn.med.nyu.edu and Christopher.Wylie@chmcc.org)

Accepted 29 May 2003

SUMMARY

In mouse embryos, germ cells arise during gastrulation and migrate to the early gonad. First, they emerge from the primitive streak into the region of the endoderm that forms the hindgut. Later in development, a second phase of migration takes place in which they migrate out of the gut to the genital ridges. There, they co-assemble with somatic cells to form the gonad. In vitro studies in the mouse, and genetic studies in other organisms, suggest that at least part of this process is in response to secreted signals from other tissues. Recent genetic evidence in zebrafish has shown that the interaction between stromal cell-derived factor 1 (SDF1) and its G-protein-coupled receptor CXCR4, already known to control many types of normal and pathological cell migrations, is also required for the normal migration of primordial germ cells. We show that in the mouse, germ cell migration and survival requires the SDF1/CXCR4 interaction. First, migrating germ cells

express CXCR4, whilst the body wall mesenchyme and genital ridges express the ligand SDF1. Second, the addition of exogenous SDF1 to living embryo cultures causes aberrant germ cell migration from the gut. Third, germ cells in embryos carrying targeted mutations in CXCR4 do not colonize the gonad normally. However, at earlier stages in the hindgut, germ cells are unaffected in CXCR4^{-/-} embryos. Germ cell counts at different stages suggest that SDF1/CXCR4 interaction also mediates germ cell survival. These results show that the SDF1/CXCR4 interaction is specifically required for the colonization of the gonads by primordial germ cells, but not for earlier stages in germ cell migration. This demonstrates a high degree of evolutionary conservation of part of the mechanism, but also an area of evolutionary divergence.

Key words: PGCs, SDF1, CXCR4, Chemokine, Migration, Mouse

INTRODUCTION

The gametes of many species arise from a small group of founder cells, the primordial germ cells. These are set aside early in development, in different regions of the embryo in different species, and often in sites far removed from the somatic cells that will form the accessory cells of the gonad. In the mouse, the primordial germ cells (PGCs) are set aside during gastrulation, when they emerge from the posterior primitive streak into the embryonic endoderm (Anderson et al., 2000; Ginsburg et al., 1990). They become incorporated into the hindgut, from which they migrate later in development to join the somatic cells of the gonad in two paired swellings of the dorsal abdominal walls, the genital ridges. Studies on cultured mouse germ cells suggested that, during migration, secreted signaling factors released by surrounding tissues control the proliferation, survival and motility of the primordial

germ cells (reviewed by Wylie, 1999). In chemotaxis assays, cultured PGCs are attracted by dissected genital ridges (Godin et al., 1990). In *Drosophila*, genetic screens for mutations that perturb germ cell migration (Moore et al., 1998) have identified proteins that mediate both repulsion (Zhang et al., 1997; Starz-Gaiano, 2001) and attraction (Van Doren et al., 1998) of the primordial germ cells during migration.

Recent work on zebrafish embryos has shown that the G-protein-coupled receptor CXCR4, and its ligand Stromal cell-derived factor-1 (SDF1; CXCL12 – Mouse Genome Informatics) (Bacon et al., 2002) play essential roles in primordial germ cell migration (Doitsidou et al., 2002; Knaut et al., 2003). The SDF1-CXCR4 interaction is known to play roles in the chemotaxis of several cell types, such as lymphocytes (Bleul et al., 1996a; Bleul et al., 1996b), cerebellar and hippocampal neurons (Lu et al., 2002; Zou et al., 1998), and lateral line cells (David et al., 2002). It also

plays a part in several pathological situations, for example, tumor metastasis (Muller et al., 2001), joint infiltration (Buckley et al., 2000) and HIV-1 entry (Feng et al., 1996). SDF1 is the only known ligand of the receptor CXCR4 (Ma et al., 1998).

In this paper we show that colonization of the gonads by PGCs in the mouse requires the ligand-receptor interaction of SDF1 and CXCR4. First, the receptor is expressed on the migrating germ cells, whereas its ligand is expressed in the dorsal body wall during migration. Second, the migration of germ cells in living embryo cultures is perturbed by the presence of added SDF1. Third, germ cells do not colonize the genital ridges correctly in embryos carrying targeted mutations of the receptor CXCR4, although the primordial germ cells do colonize the hindgut in these embryos. In addition, addition of SDF1 causes increased survival of germ cells, whereas targeted mutation of the CXCR4 receptor causes a progressive reduction of germ cells during and after the colonization of the genital ridges.

These data show that the SDF1-CXCR4 interaction is needed for the growth and survival of germ cells, and that this interaction may also play a role in guiding migrating germ cells to the genital ridge. The fact that germ cell development is affected in CXCR4-mutant mice and zebrafish further suggests that the same G-protein-coupled receptor (GPCR)-signaling mechanism regulates early germ cell development in vertebrates.

MATERIALS AND METHODS

Organ culture

Transverse slices from the hindgut regions of mouse embryos were cultured and filmed as previously described (Molyneux et al., 2001), with the following modifications. Previous experiments were performed in DMEM/F-12 (Gibco BRL) medium with 15% serum. To analyze the effect of SDF1 on PGC migration, slices were incubated in DMEM/F-12 medium with 0.4 mg/ml lipid free BSA (Sigma), 100 U/ml penicillin and 100 U/ml streptomycin (organ culture media). SDF1 (Sigma) was added at the indicated concentrations. Slices were filmed using the Zeiss LSM 510 confocal system. Images were captured every 7 minutes for 7.5-11 hours. Movies were analyzed using NIH image as previously described. For bead experiments, heparin coated beads (Sigma) were washed three times in PBS and then incubated for 1 hour in the indicated concentrations of SDF1, or in 1 mg/ml lipid-free BSA. Beads were washed twice for 5 minutes in PBS and then once in organ culture media. Beads were placed into the aorta of slices using tungsten needles. For counting PGCs in slices, the tissue was optically sectioned in 10 µm steps (with 5 µm overlap). PGCs were counted in individual optical sections by using the overlay feature in the Zeiss software to mark cell positions.

RT-PCR and chip analysis

PGC containing tissue was dissected from E10.5, E11.5 and E12.5 animals, and digested in 500 µl of 0.25% trypsin (37°C for 15 minutes). The tissue was triturated into a single cell suspension and filtered through a nylon mesh. The mesh was washed with 1 ml 2% BSA in PBS and the resulting 1.5 ml suspension was sorted using a FACS Vantage. GFP-positive cells (typically 98% pure) or GFP-negative cells were spun down (10 minutes at 1,000 g) and lysed in 300 µl TriZol Reagent (Invitrogen). RNA was isolated according to the manufacturer's instructions, using 5 µg linear polyacrylamide (Sigma) as a carrier. For quantitative RT-PCR, 15 ng of PGC or

somatic mRNA was reverse transcribed in a 10 µl volume containing 1×buffer (Invitrogen), 100 ng Oligo dT, 2 mM DTT, 0.5 mM dNTPs, 10 U RNasin (Promega), 200 U Superscript II (Invitrogen) and 8 µg T4gp32 protein (USB). Standard curves were generated by diluting PGC or somatic cDNAs 1:10, 1:20 and 1:100 in H₂O. 1 µl of the dilutions was used for PCR. PCR was performed in a 25 µl volume using QuantiTect SYBR Green mix (Qiagen) as a source of Taq, buffer and dNTPs. PCR mix was supplemented with 5% DMSO, as per MJ Research instructions for difficult templates. Cycling and quantitation was performed in the Opticon Cycler (MJ Research) (15 minutes at 95°C; followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 51°C, 40 seconds at 72°C and 30 seconds at 73°C [optical read]; followed by a 5 minute extension at 72°C and a melting curve). Primers were used at a final concentration of 0.2 µM and were as follows: CXCR4, AGCCTGTGGATGGTGGTGTTC (forward) and CCTTGCTTGATGACTCCCAAAG (reverse);

GAPDH, ACCACAGTCCATGCCATCAC (forward) and TCCAC-CACCCTGTTGCTGTA (reverse);

ODC, GCCATTGGGACAGGATTTGAC (forward) and CATCA-TCTGGACTCCGTTACTGG (reverse);

Stag3, AGTGGGCAAGAAGCAAAAAGG (forward) and TTCC-ATAAGGCTGAGTCGGGTC (reverse);

SPARC, AAGATACTGTGAGACTGAGGACCC (forward) and TGGAAAGAAACGCCCGAAG (reverse); and

Cystatin C, CAACAAGGGCAGCAACGATG (forward) and GGGAAAGGAGCACAAGTAAGGAAC (reverse).

For chip analysis, 15 ng RNA was reverse transcribed as above except 100 ng Oligo-dT T7 (GTAATACGACTCACTATAGGGCT₍₁₈₎) and 1 µl SMARTII Oligo (Clontech) were added to the reaction. Half of the resulting cDNA was amplified using the Smart cDNA synthesis kit (Clontech) with Oligo-dT-T7 as a 3' primer. Amplified cDNA was purified over a QIAquick column (Qiagen), and precipitated with sodium acetate and ethanol. Probes were prepared from amplified cDNA using the BioArray Labeling kit (Enzo). Probes were applied to MG-U74Av2 chips (Affymetrix) according to the manufacturer's instructions. Chip analysis was performed using MicroArray Suite software (Affymetrix) to statistically determine 'presence' and 'absence' calls. The average chip signal was normalized to an arbitrary value of 1000.

Immunostaining

Slices were fixed in 4% PFA/PBS for 20 minutes at room temperature. Tissues were washed for five minutes with PBS (3×) and stored overnight in PBS with 0.1% TX-100 (4°C, with rocking). Slices were blocked (overnight, 4°C) in 2% Donkey Serum in PBS (blocking buffer). Anti-SDF1 (Sigma) was used at 2.5 µg/ml in blocking buffer (overnight, 4°C). Tissues were washed five times for 1 hour in PBS/0.1% TX-100 at room temperature. Cy5-donkey anti-goat (Jackson Immuno Research) was used at 15 µg/ml in blocking buffer (overnight, 4°C). Slices were washed as above and mounted in 75% glycerol on Lab-Tek chambered coverglass (NalgeNunc).

Mouse breeding and embryo preparation

All animals were treated according to protocols approved by the Committee on Animal Research at New York University School of Medicine. Embryos from E9.5-E12.5 were recovered from matings between two CXCR4^{+/-}-mutant animals (Zou et al., 1998). CXCR4^{+/+}, ^{+/-} and ^{-/-} embryos were fixed overnight at 4°C in 4% formalin (Ultra pure formaldehyde, Polysciences). Embryos were then washed with PBS, partially dissected and incubated in 30% sucrose overnight at 4°C. The posterior region was mounted in OCT compound (Tissue-Tek) and frozen in dry ice. Blocks were kept at -80°C until processing. All cryosections presented in this paper were 20 µm thick.

Alkaline phosphatase staining of germ cells

Sections on slides were washed in PBS then in alkaline phosphatase (AP) buffer (100 mM NaCl, 100 mM Tris PH 9.5, 50 mM MgCl₂,

0.1% Tween) for 10 minutes. The sections were stained with BCIP/NBT (0.33 mg/ml and 0.2 mg/ml, respectively) in AP buffer at RT for 20 minutes. Stained sections were mounted using Crystal Mount (Biomed). Pictures were taken on an Axioskop microscope (Zeiss) and images acquired via a JVC digital KY-F70 camera and saved as 24-bit Tiff files in Adobe Photoshop. All sections were photographed with a 5× objective.

Genotyping for CXCR4

Genomic DNA was prepared from yolk sacs or tissues in SDS-free buffer at 55°C [50 mM KCl, 10 mM Tris (pH 8.5), 0.01% gelatin, 0.45% Nonidet P-40, 0.45% Tween-20]. Proteinase K was added to a final concentration of 200 µg/ml.

The sequences of the primers used to detect both a wild-type (1 kb) and a mutant (450 bp) band are given below:

5'-TGGCTGACCTCCTCTTTGTCATCA-3'

5'-TGGAGTGTGACAGCTTGGAGATGA-3'

RESULTS

CXCR4 is expressed in PGCs during the migratory and postmigratory stages

To determine whether CXCR4 and SDF1 play roles in mouse germ cell behavior, we first analyzed their expression patterns using RNA profiling and protein expression. Migratory PGCs were purified from E10.5 embryos and post-migratory PGCs were purified from E12.5 embryos using FACS. For Affymetrix chip analysis, PGC cDNA (from ~2000 cells) was amplified using the Clontech Smart cDNA synthesis kit and 24 cycles of PCR. Two independent E10.5 samples and two E12.5 samples were applied to the MG-U74Av2 array (12,488 features). On the array, CXCR4 was found to be present at similar levels in E10.5 and E12.5 PGCs (Fig. 1A). This observation was confirmed by RT-PCR using unamplified PGC cDNA (Fig. 1B). Additionally, we have used quantitative RT-PCR to demonstrate that CXCR4 is enriched in PGCs relative to the somatic tissue of the gonad (Fig. 1C).

The distribution of SDF1 protein was examined at E9.5 by whole-mount immunostaining. SDF1 was found to be present throughout the entire dorsal body wall, with areas of higher staining in the floor plate of the neural tube, and in the mesonephros and adjacent mesenchyme (Fig. 1D,E). McGrath et al. showed that *Sdf1* RNA is expressed in the region surrounding the aorta, gonad and the mesonephros of the E10.5 embryo (McGrath et al., 1999). The mRNA and protein

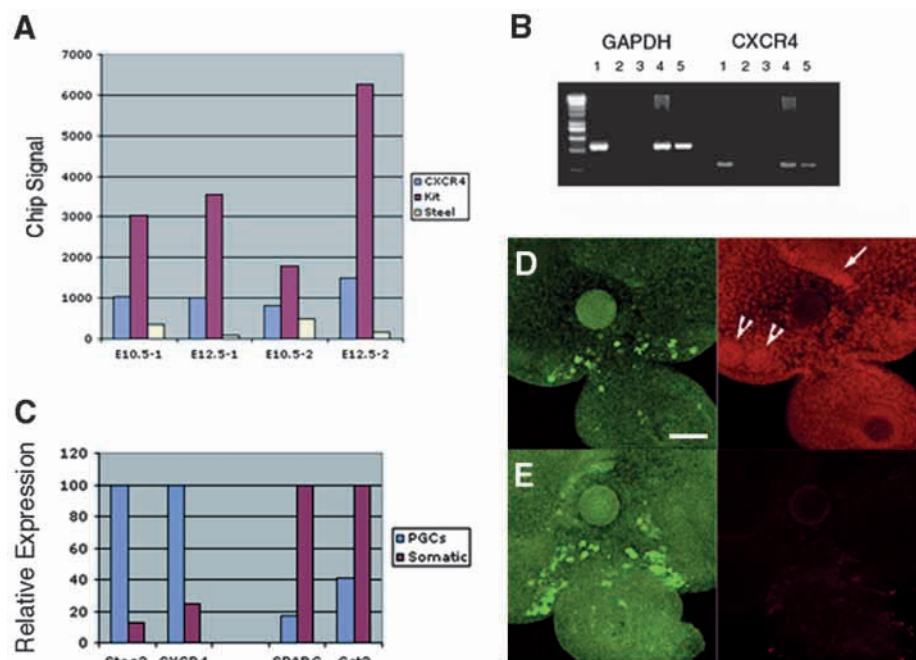


Fig. 1. CXCR4 is expressed in PGCs during and after colonization of the gonad. (A) Probes prepared from E10.5 (two biological replicates) and E12.5 (two biological replicates) PGCs were applied to Affymetrix chips. Chip data was analyzed using MicroArray Suite v5.0 software (Affymetrix) to generate signal intensity data, and to statistically determine presence and absence calls. The average chip signal was normalized to an arbitrary value of 1000. The bar graph shows the signal intensity of the CXCR4 probe set for each sample. CXCR4 was called present in all samples. The PGC marker gene *Kit* was also called present in all samples. The somatic marker gene *Steel* (*Kitl* – Mouse Genome Informatics) was called absent. (B) CXCR4 expression was confirmed by RT-PCR. Lane 1, E10.5 PGC cDNA; Lane 2, RT–; Lane 3, H₂O blank; Lane 4, E12.5 PGC cDNA; Lane 5, E10.5 whole-embryo cDNA. (C) *Cxcr4* message is enriched in PGCs relative to the somatic tissue. The level of *Cxcr4* transcripts in E11.5 PGCs (GFP+) or somatic tissue (GFP–) were quantified by SYBR-green based RT-PCR. The meiotic marker, *STAG3* (Pezzi et al., 2000) was used as a positive control for PGCs, and the gonadal markers *SPARC* and *cystatin C* (*CST3*) (Wertz and Herrmann, 2000) were used as positive controls for the somatic component of the gonad. For CXCR4 and *STAG3*, the PGC cDNA was used to generate a standard curve and expression in this tissue was set to an arbitrary value of 100. For *SPARC* and *CST3*, the somatic tissue cDNA was used to generate standard curves. Expression was normalized to ODC levels in the somatic and PGC samples. (D) SDF1 is expressed in the genital ridge area. Anti-SDF1 staining is shown in red. This slice was taken from an E9.5 +/Oct4ΔPE:GFP+ embryo. A BSA-coated bead was placed in the aorta and the slice was cultured for 12 hours, then fixed and stained with 2.5 µg/ml anti-SDF1 antibody. Arrows indicate the region of more intense SDF1 staining in the floor plate of the neural tube, and arrowheads indicate the mesonephros and adjacent mesenchyme. The germ cells are marked by bright green GFP fluorescence. (E) Control for SDF1 staining. This control slice was treated in the same way as the experimental slice in D, but the anti-SDF1 antibody was omitted. Scale bar in D: 82 µm for D,E.

expression patterns demonstrate that SDF1 is expressed at this time, and that the protein is enriched in the genital ridge region.

SDF1 alters PGC migration in slice cultures

To determine whether added SDF1 could affect PGC behavior, we cultured transverse slices from the hindgut regions of E9.5 embryos for 20 hours in serum-free medium, either in the presence or absence of added SDF1. PGC behavior was observed in the slices using the Oct4ΔPE:GFP+ transgene, which allows direct observation of the living PGCs (Anderson et al., 2000). In the absence of SDF1, germ cells emerged from the hindgut, exclusively from its dorsal aspect, and divided into

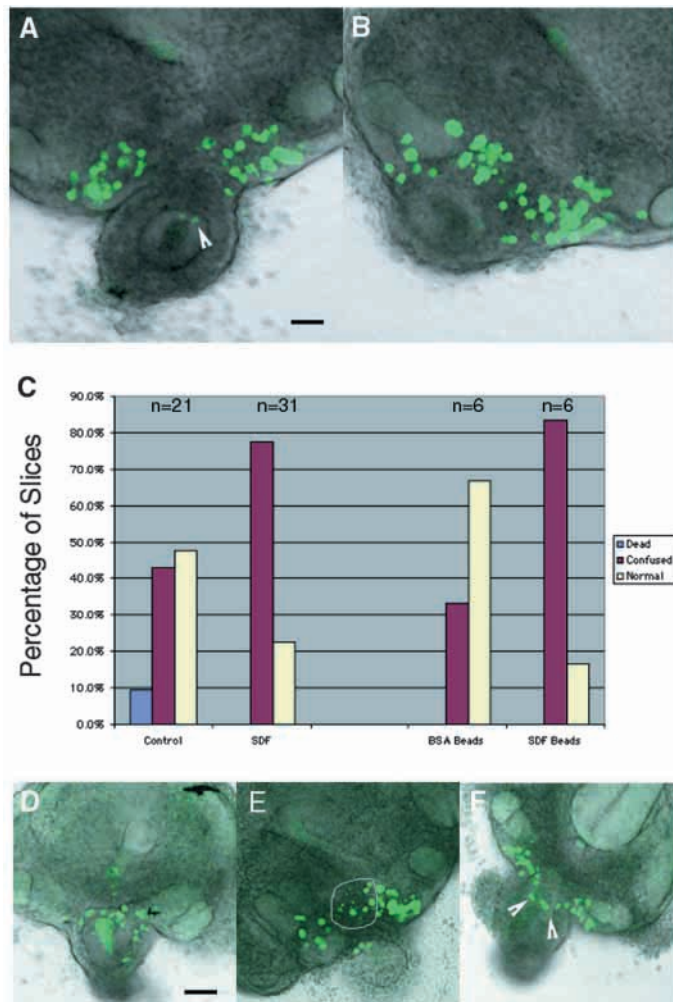


Fig. 2. SDF1 alters PGC behavior in an organ culture assay. (A) A slice taken from an E9.5 +/Oct4 Δ PE:GFP⁺ embryo after 20 hours in culture. PGCs (green) have formed clusters at the genital ridges. A few cells (arrowhead) remain in the hindgut. (B) An E9.5 slice incubated for 20 hours in the presence of 1.6 μ g/ml SDF1. Some PGCs have colonized the genital ridges, but many cells remain scattered across the midline. (C) Summary of migration data from four culture experiments. Slices were incubated in the presence of a range of SDF1 concentrations (5 ng/ml to 1 μ g/ml). PGC migration was affected at all concentrations (data not shown). Beads coated with 50 μ g/ml SDF1 caused a similar effect to treatment with soluble SDF1. (D) An example of a control slice that was scored as dead. The majority of cells remain trapped in the hindgut. (E) Example of a control slice scored as confused. The circled cells have failed to clear the midline. (F) Example of a control slice scored as normal. The midline of the body wall is clear and only a few PGCs (arrowheads) remain in the hindgut. Scale bars: in A, 46 μ m for A,B; in D, 68 μ m for D-F.

two bilateral streams, which migrated laterally towards the genital ridges (Fig. 2A). At the end of the culture period, germ cells had formed discrete groups in each genital ridge in 10 out of 21 slices (49%); in 9 out of 21 slices (42%), germ cells remained more scattered across the midline. In a small percentage of slices, germ cells failed to migrate significantly (2/21). This behavior contrasts with that observed previously in high concentrations of serum (Molyneaux et al., 2001), in

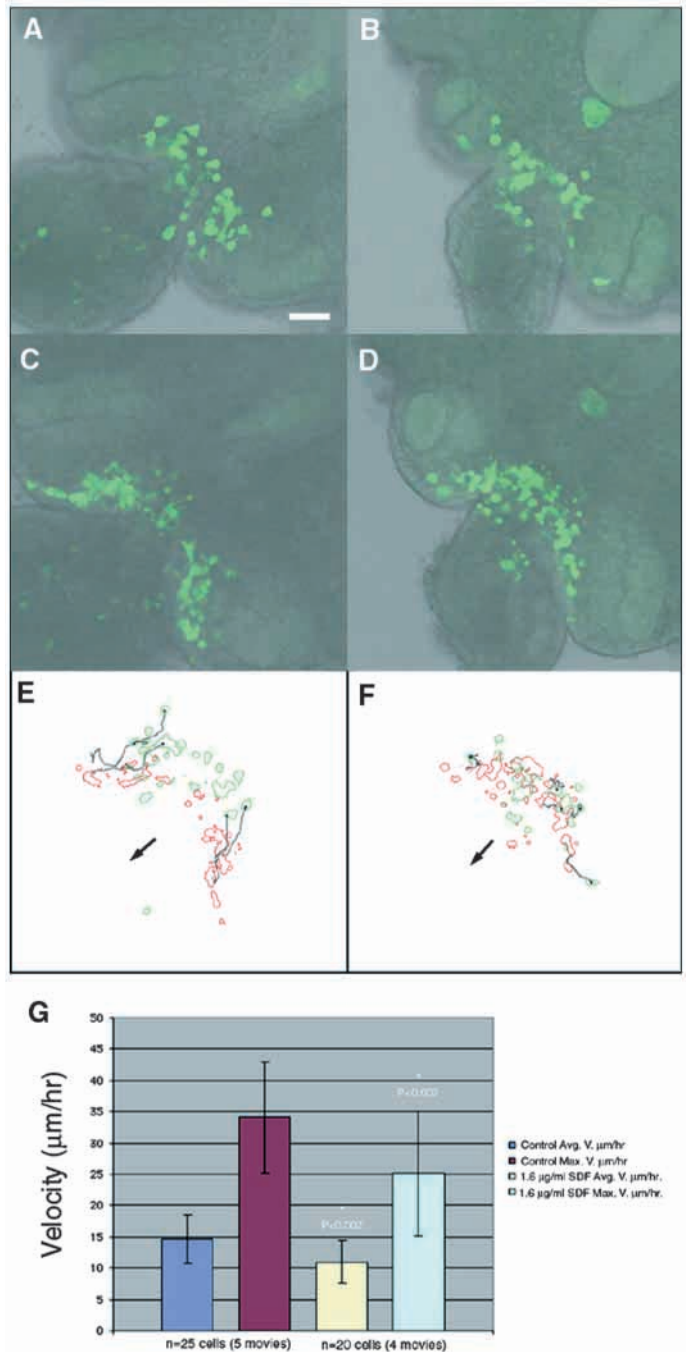


Fig. 3. Soluble SDF1 decreases the average and maximum velocity of PGCs. (A) An E9.5 control slice. (B) An E9.5 slice in the presence of 1.6 μ g/ml SDF1. (C,D) Slices shown in A,B after 7.5 hours of culture, respectively. (E,F) Trajectories of cells in control (E) and SDF1-treated (F) slices. Green outlines represent starting positions of cells and red outlines indicate ending positions of cells. Arrow indicates the position of the gut. Black lines follow trajectories of five selected cells in the experimental and control slices. (G) Summary of PGC velocity data from five control and four SDF1-treated slices. Error bars show standard deviation. Scale bar: 55 μ m (A-F).

which PGCs did not migrate directionally in the body wall mesenchyme, which leads us to conclude that there are inhibitory factors in serum.

Addition of SDF1 to the medium reduced the lateral migration of the PGCs (Fig. 2B). PGCs emerged from the hindgut, but remained scattered near the midline in 24 out of 31 slices (77%). The effect was the same across the dose ranges used (50 ng/ml to 1.6 μ g/ml; data not shown). SDF1-coated beads, placed in the midline aorta, also caused the same effect (PGCs moved laterally to form bilateral clusters in only one out of six slices, compared with four out of six slices containing BSA-coated control beads). These experiments suggest that high concentrations of SDF1, either uniformly or ectopically applied, alter the ability of PGCs to migrate normally in the body wall. Fig. 2C summarizes this data, which was accumulated from four experiments, and Fig. 2D-F shows examples of slices in which lateral migration was scored as normal or abnormal. A slice was scored as 'dead' if the majority (>50%) of PGCs remained in the hindgut; as 'confused' if any PGCs remained on the midline of the body wall; or as normal if the PGCs exited the gut and cleared the midline.

Soluble SDF1 slows PGC migration

To test whether the PGC migration defect caused by added SDF1 was caused by a change in motility, slices were incubated in the presence or absence of soluble SDF1, and the GFP-marked PGCs were filmed for 7.5 hours starting at E9.5. Fig. 3A-D shows examples of beginning and endpoint pictures taken from control (Fig. 3A,C) and SDF1-treated slices (Fig. 3B,D) (see Movies at <http://dev.biologists.org/supplemental/>). The movements of five cells were traced in each movie and the resulting traces are shown in Fig. 3E,F. Cells in the SDF1-treated slice had short and twisted trajectories (Fig. 3F). Similar results were seen in four slices. Velocity data for these are presented in Fig. 3G. SDF1 treatment produced a statistically significant (Student's *t*-test) decrease in both the average and maximum velocity of PGCs, showing that PGC motility is decreased by elevated concentrations of SDF1.

SDF1-coated beads attract PGCs and shorten their trajectories

To determine if ectopically applied SDF1 can attract PGCs, SDF1-coated beads were placed into the aortas of E9.5 slices and the slices were filmed for 11 hours to observe PGC behavior. Fig. 4A-D shows examples of cell trajectories in SDF1-treated and control slices. BSA-coated beads produced no effect on PGC migration (Fig. 4A). Cells in these slices had long, straight trajectories and the majority of cells moved towards the developing genital ridges. Similar behavior was observed in three control slices. However, SDF1-coated beads confused and 'captured' PGCs. Whereas some PGCs moved towards the genital ridges, PGCs that started nearest to the bead moved towards it and exhibited twisted (zig-zag) trajectories around the bead. Movies of the control experiment (Fig. 4A) and the 50 μ g/ml experiment (Fig. 4D) are available (see Movies at <http://dev.biologists.org/supplemental/>). PGC capture appeared to depend on the dose of SDF1 supplied by the bead. To quantify this effect, 5 cells were tracked in each

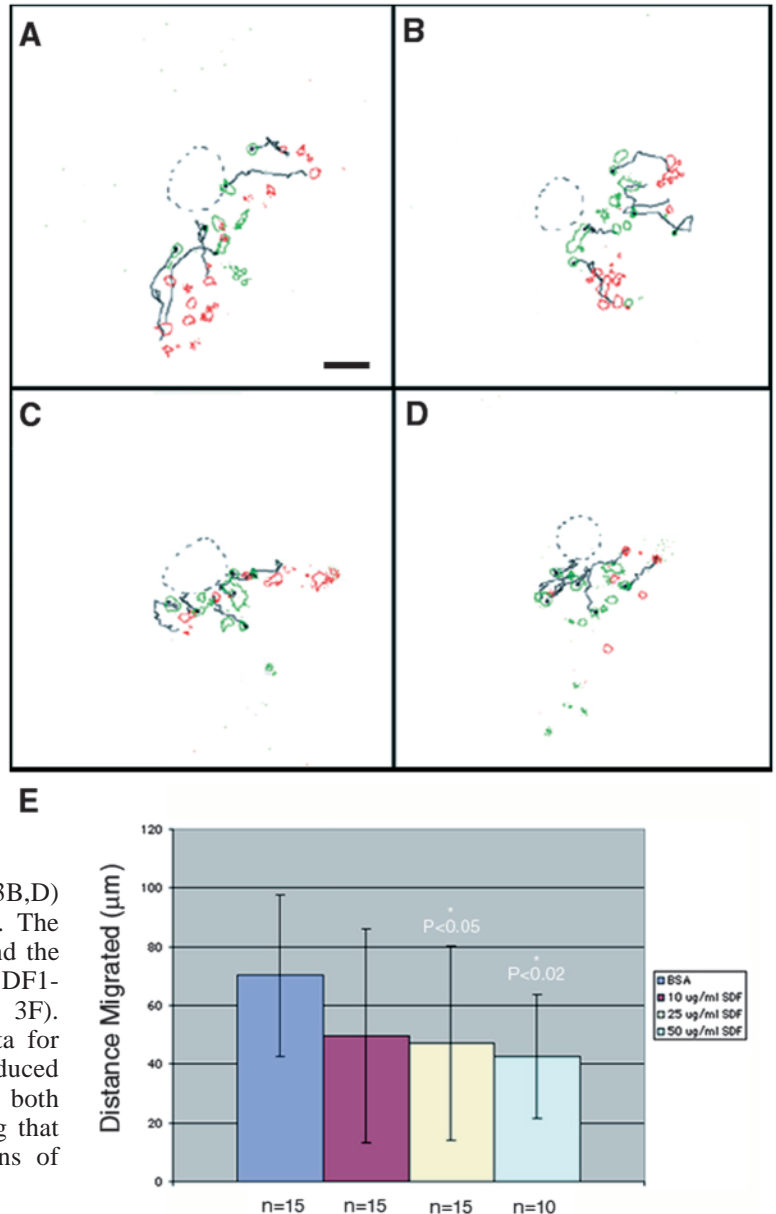


Fig. 4. SDF1-coated beads capture migrating PGCs. (A) Trajectories of cells in a control slice. A BSA-coated bead was placed into the aorta of an E9.5 slice and the slice was filmed for eleven hours. Green outlines represent starting positions of cells and red outlines represent ending positions. A dotted outline indicates the position of the bead. (B-D) Trajectories of cells near beads coated with 10 (B), 25 (C) and 50 (D) μ g/ml SDF1. (E) Summary of migration data from control and SDF1-coated bead experiments. Movies of three slices per treatment were analyzed, except for the 50 μ g/ml treatment where movies of two slices were analyzed (see Movies at <http://dev.biologists.org/supplemental/>). Error bars show standard deviation. Scale bar: 55 μ m (A-D).

slice and the total distance that each cell migrated was recorded (Fig. 4E). Cells in control slices moved an average of 70.2 \pm 27.6 μ m. However, at high doses of SDF1, 25 μ g/ml and 50 μ g/ml, trajectories were shortened to 47.2 \pm 33.2 μ m and 42.2 \pm 21.1 μ m, respectively. Unlike soluble SDF1, SDF1 on

beads did not have a statistically significant effect on either the average or maximum velocity of PGCs (data not shown). These data suggest that at short range, high local concentrations of SDF1 can attract migrating germ cells and alter their trajectories.

The 50 $\mu\text{g/ml}$ movies were examined to estimate the range of the SDF1 effect. Of the 10 cells tracked in these films, six exhibited twisted trajectories, whereas four exhibited more normal behavior. The starting distance from the bead was recorded for each cell. On average, cells exhibiting twisted trajectories started near the bead ($19.2 \pm 17.2 \mu\text{m}$ away). Cells with more normal trajectories started farther from the bead ($37.0 \pm 16.8 \mu\text{m}$). From this we estimate that the range of the effect is 20–40 μm .

CXCR4-mutant embryos show defects in germ cell migration and survival

To determine the role of SDF1 and its receptor CXCR4 during germ cell migration we analyzed CXCR4-deficient embryos during various stages of germ cell development. Mutant embryos were compared with control embryos during and after migration, from E9.5 to E12.5. During this time PGCs in CXCR4^{+/+} or CXCR4^{+/-} control embryos leave the hindgut and migrate dorsally, separate into two bilateral groups, and move into the genital ridges. CXCR4-mutant embryos could not be distinguished from their siblings at E9.5 (data not shown), which suggests that the specification of germ cells and their initial migration into, and then within, the developing gut, do not require CXCR4. Between E9.5 and E10.5 wild-type PGCs migrate from the hindgut towards the bilateral genital ridges. In E10.5 mutant sibling embryos, we detected less germ cells in the genital ridge, and more germ cells along the mesentery and the hindgut compared with wild type (Fig. 5A,B). Shortly thereafter, at E11.5, most control PGCs have entered the genital ridge and the total number of germ cells has increased in wild-type embryos. However, CXCR4-deficient embryos showed a striking decrease of PGCs in the genital ridge compared with wild-type or heterozygous siblings at this stage (Fig. 5C,D). This defect can only partially be explained by a delay in migration, as the overall number of PGCs in the genital ridge and the mesentery was reduced at this stage in the mutant compared with control embryos. Thus CXCR4 seems to be required for the proliferation and/or survival of germ cells. In normal embryos, PGCs in the genital ridge continue to divide, and increase in number from less than 100 at the onset of migration to more than 25,000 at stage E13 (Tam and Snow, 1981). We have quantified the PGC survival defect in CXCR4-mutant animals by counting PGCs in tissue sections (Fig. 5G). Beginning at stage E10.5, CXCR4-mutant embryos had less PGCs in the genital ridge compared with their heterozygous and wild-type siblings. By E12.5, the number of PGCs in both mutant and wild-type embryos increased by approximately the same amount (Fig. 5E,F,G), suggesting that CXCR4 is not required for germ cell survival and/or proliferation once PGCs reach the genital ridge. These data, together with the slice culture results, suggest that CXCR4 function in germ cells may initially be required to mediate the directed migration of germ cells towards the genital ridge; however, CXCR4 and SDF1 may also control germ cell survival during migration.

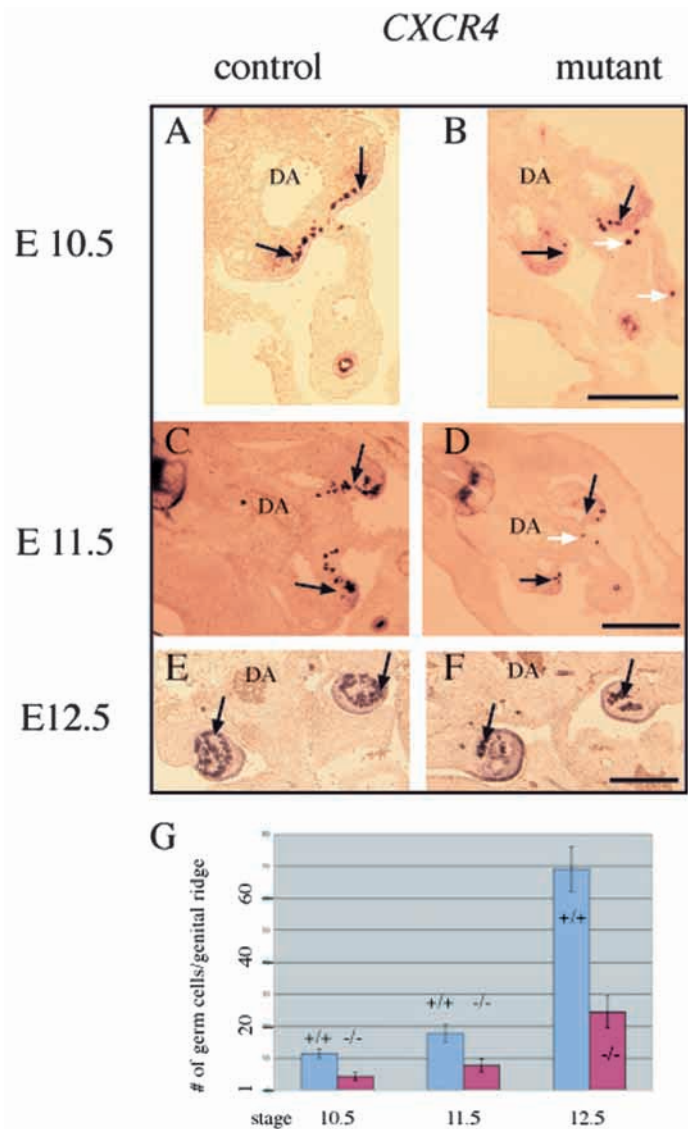


Fig. 5. Reduction in the number of PGCs reaching the genital ridge in CXCR4-mutant embryos. Cryosections of E10.5 (A,B), E11.5 (C,D) and E12.5 (E,F) control (+/+, A,C; +/-, E) and mutant (-/-, B,D,F) embryos. PGCs are stained with AP: black arrows indicate PGCs in the genital ridge and white arrows point to lost PGCs in mutant embryos. DA indicates position of dorsal aorta in each section. Scale bars: 0.5 mm. (G) Number of PGCs in control and mutant embryos. For each bar, PGCs were counted in eight transverse 20 μm sections distributed along the genital ridge. Error bars show standard deviation. There is a statistically significant difference in the number of PGCs between control and mutant embryos (Student's *t*-test, $P < 0.0001$).

SDF1 enhances PGC survival in slice cultures

The reduced number of PGCs seen in CXCR4-deficient mice suggests that SDF1 might be required for PGC survival, as well as migration. To test whether SDF1 alters PGC survival, PGC-containing tissue slices from E10.5 embryos were cultured in the presence or absence of SDF1. Cells in each slice were counted by optically sectioning the slice. Fig. 6A–D shows examples of a control and SDF1-treated slice, before and after

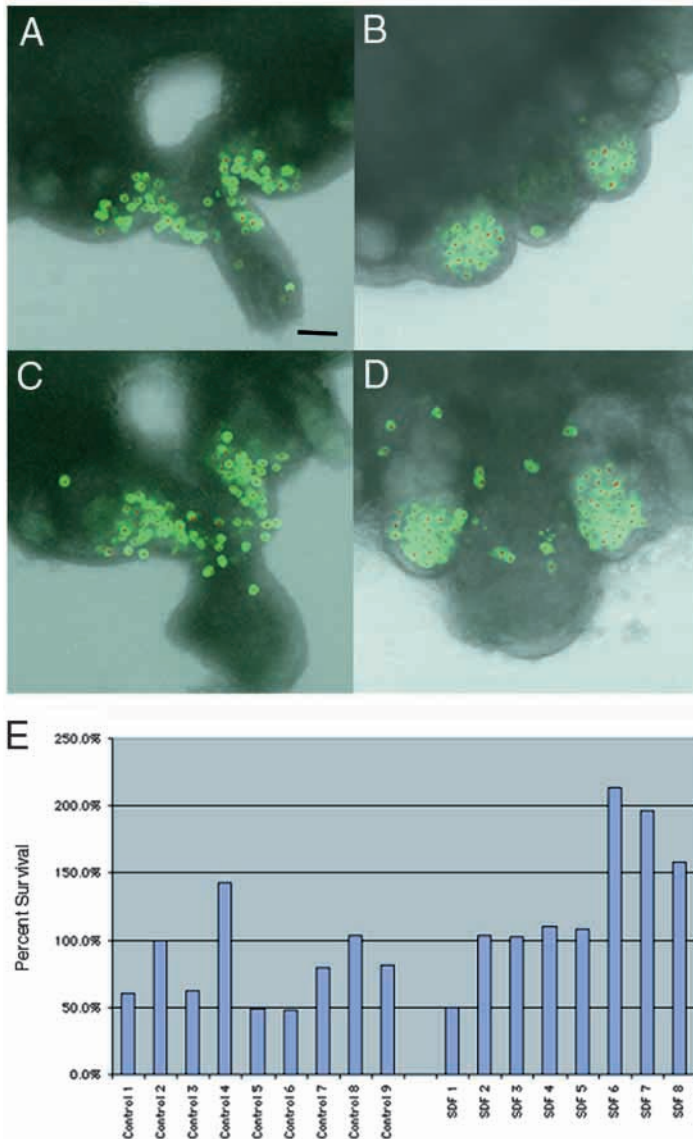


Fig. 6. Soluble SDF1 enhances PGC survival in E10.5 slices. (A) A control slice before culture. This slice was optically sectioned and the picture shown is a projection ($z=156.4 \mu\text{m}$). Red marks indicate counted cells. (B) The slice shown in A after 20 hours in culture. The picture shown is a projection ($z=83.7 \mu\text{m}$). Slices compress while in culture. (C) An SDF1-treated slice prior to culture ($z=150.0 \mu\text{m}$). (D) The slice shown in C after 20 hours in the presence of 500 ng/ml SDF1 ($z=76.8 \mu\text{m}$). (E) Summary of survival data from two culture experiments. The slice shown in A is Control 3 in the graph, and the slice shown in C is SDF1 5. Most control slices have poor survival, whereas SDF1 treatment enhances survival and/or proliferation. Scale bar: 55 μm (A-D).

20 hours in culture. Fig. 6E summarizes the survival data from two experiments. PGC numbers in control slices declined (average of 80.7% survival), whereas, PGC numbers in SDF1-treated slices showed a statistically significant increase (average of 130.4% survival; Student's *t*-test, $P < 0.03$). This finding suggests that SDF1 acts as a PGC growth or survival factor.

DISCUSSION

The data presented here show that SDF1-CXCR4 interaction is required for the normal colonization of the genital ridges by the primordial germ cells, and their subsequent accumulation into sex cords. SDF1 added to slice cultures of embryos during migration caused defective movements and increased numbers of germ cells. SDF1-treated beads diverted germ cells closest to them from their normal migratory route. Embryos homozygous for a targeted mutation in the CXCR4 receptor showed dramatically reduced numbers of germ cells colonizing the genital ridges. The CXCR4 mutation did not block the colonization of the endoderm by germ cells, nor their migration from the hindgut. This suggests that SDF1-CXCR4 interaction is required specifically for germ cell behavior after they have left the hindgut and are migrating through the mesenchyme to the genital ridges. In addition, some germ cells did colonize the genital ridges in CXCR4^{-/-} embryos, which suggests that other migratory and locomotory cues, in addition to SDF1, may play roles in germ cell migration. Several possibilities would explain the observations. First, a primary role for SDF1 could be in proliferation or survival. In the absence of CXCR4/SDF1 the population of germ cells could become progressively smaller, so that fewer germ cells colonize the genital ridges, and these eventually die. Second, the primary role could be motility. We have shown previously that germ cells furthest from the genital ridges die (Molyneux et al., 2001). Lack of SDF1 signaling might slow down the germ cells. Then, as the distance to the genital ridge increases (the embryo grows twofold in size between E9.5 and E11.5), more and more germ cells would be outside the range of survival signals from the genital ridges, and would die. Third, the primary role could be directionality. Germ cells lacking the normal directional cues could end up too far away from the genital ridges and die. Data from slice cultures show that motility, directionality and numbers of germ cells, are all altered by addition of ectopic, or increased amounts of, SDF1, suggesting that there is more than one downstream consequence of activation of the CXCR4 receptor.

The experiments with SDF1-coated beads show that germ cells closest to the bead (i.e. within a few cell diameters) move to, or remain around, the bead, whereas those further away ignore the bead and migrate laterally towards the genital ridges. This suggests either that the chemoattractive range of SDF1-coated bead is small, or that it is countered, at greater distances, by the signal provided by the endogenous environment. During migration germ cells extend long processes (see Movies at <http://dev.biologists.org/supplemental/>) that span at least 2-4 cell diameters. A contact-based mechanism, as previously suggested as a basis for the action of SDF1 on migrating lateral line cells (David et al., 2002), may therefore also account for the narrow range of effect of SDF1 on germ cells.

It is interesting to compare the roles of SDF1/CXCR4 in the mouse with those observed in zebrafish. It is clear that neither species require this ligand-receptor pair for germ cell formation. However, in the zebrafish, mutations or oligo-mediated loss-of-function cause defective migration from its onset (Doitsidou et al., 2002; Knaut et al., 2003), whereas, in the mouse, CXCR4 is dispensable for the early migration of

PGCs. In the mouse, the early migration is from the posterior primitive streak (E7.5) into the hindgut endoderm, where they remain actively motile until E9.5, when they exit dorsally from the hindgut. The SDF1-CXCR4 interaction is not required for this component of migration. This component of germ cell migration is not shared by the zebrafish, which may explain why it is not mediated by a common signaling pathway. In the zebrafish, ectopic migration of germ cells can be easily seen, suggesting that SDF1-CXCR4 interaction is not required for survival, whereas, in the mouse, the numbers of germ cells are increased by the addition of SDF1 and decreased in CXCR4^{-/-} embryos.

In conclusion, we have shown that the SDF1-CXCR4 interaction is required for normal colonization of the gonad by PGCs in early mouse embryos. We also show that part of the PGC migration process in the mouse shares a conserved molecular mechanism with the zebrafish, whereas other parts show evolutionary divergence.

We thank Gord Fishell for support with microscopy and dissection; Dan Marmer for his assistance in sorting PGCs; and Sara Rankin and Shawn Smith for their help with the chip experiments. J.S. is supported by the Swiss National Science foundation and E.R. is supported by the DFG and the Volkswagen-Stiftung. K.A.M. is supported by an NRSA fellowship (1 F32 HD008678-01) from the NIH. Funding support was also provided by R01 HD33440-01 awarded to C.W. R.L. is an investigator of the Howard Hughes Medical Institute.

REFERENCES

- Anderson, R., Copeland, T. K., Scholer, H., Heasman, J. and Wylie, C. (2000). The onset of germ cell migration in the mouse embryo. *Mech. Dev.* **91**, 61-68.
- Bacon, K., Baggolini, M., Broxmeyer, H., Horuk, R., Lindley, I., Mantovani, A., Maysushima, K., Murphy, P., Nomiyama, H., Oppenheim, J. et al. (2002). Chemokine/chemokine receptor nomenclature. *J. Interferon Cytokine Res.* **22**, 1067-1068.
- Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Soderroski, J. and Springer, T. A. (1996a). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* **382**, 829-833.
- Bleul, C. C., Fuhlbrigge, R. C., Casasnovas, J. M., Aiuti, A. and Springer, T. A. (1996b). A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J. Exp. Med.* **184**, 1101-1109.
- Buckley, C. D., Amft, N., Bradfield, P. F., Pilling, D., Ross, E., Arenzana-Seisdedos, F., Amara, A., Curnow, S. J., Lord, J. M., Scheel-Toellner, D. et al. (2000). Persistent induction of the chemokine receptor CXCR4 by TGF-beta 1 on synovial T cells contributes to their accumulation within the rheumatoid synovium. *J. Immunol.* **165**, 3423-3429.
- David, N. B., Sapede, D., Saint-Etienne, L., Thisse, C., Thisse, B., Dambly-Chaudiere, C., Rosa, F. M. and Ghysen, A. (2002). Molecular basis of cell migration in the fish lateral line: role of the chemokine receptor CXCR4 and of its ligand, SDF1. *Proc. Natl. Acad. Sci. USA* **99**, 16297-16302.
- Doitsidou, M., Reichman-Fried, M., Stebler, J., Kopranner, M., Dorries, J., Meyer, D., Esguerra, C. V., Leung, T. and Raz, E. (2002). Guidance of primordial germ cell migration by the chemokine SDF-1. *Cell* **111**, 647-659.
- Feng, Y., Broder, C. C., Kennedy, P. E. and Berger, E. A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872-877.
- Ginsburg, M., Snow, M. H. L. and McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development* **110**, 521-528.
- Godin, I., Wylie, C. C. and Heasman, J. (1990). Genital ridges exert long-range effects on mouse primordial germ cell numbers and direction of migration in culture. *Development* **108**, 357-363.
- Knaut, H., Werz, C., Geisler, R. and Nusslein-Volhard, C. (2003). A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. *Nature* **421**, 279-282.
- Lu, M., Grove, E. A. and Miller, R. J. (2002). Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. *Proc. Natl. Acad. Sci. USA* **99**, 7090-7095.
- Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T., Bronson, R. T. and Springer, T. A. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc. Natl. Acad. Sci. USA* **95**, 9448-9453.
- McGrath, K. E., Koniski, A. D., Maltby, K. M., McGann, J. K. and Palis, J. (1999). Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Dev. Biol.* **213**, 442-456.
- Molyneaux, K. A., Stallock, J., Schaible, K. and Wylie, C. (2001). Time-lapse analysis of living mouse germ cell migration. *Dev. Biol.* **240**, 488-498.
- Moore, L., Broihier, H., VanDoren, M., Lunsford, L. and Lehmann, R. (1998). Identification of genes controlling germ cell migration and embryonic gonad formation in *Drosophila*. *Development* **125**, 667-678.
- Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N. et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**, 50-56.
- Pezzi, N., Prieto, I., Kremer, L., Perez Jurado, L. A., Valero, C., Del Mazo, J., Martinez, A. C. and Barbero, J. L. (2000). STAG3, a novel gene encoding a protein involved in meiotic chromosome pairing and location of STAG3-related genes flanking the Williams-Beuren syndrome deletion. *FASEB J.* **14**, 581-592.
- Starz-Gaiano, M., Cho, N. K., Forbes, A. and Lehmann, R. (2001). Spatially restricted activity of a *Drosophila* lipid phosphatase guides migrating germ cells. *Development* **128**, 983-991.
- Tam, P. P. and Snow, M. H. (1981). Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. *J. Embryol. Exp. Morphol.* **64**, 133-147.
- Van Doren, M., Broihier, H., Moore, L. and Lehmann, R. (1998). HMG-CoA reductase guides migrating primordial germ cells. *Nature* **396**, 466-469.
- Wertz, K. and Herrmann, G. (2000). Large-scale screen for genes involved in gonad development. *Mech. Dev.* **98**, 51-70.
- Wylie, C. (1999). Germ Cells. *Cell* **96**, 165-174.
- Zhang, N., Zhang, J., Purcell, K., Cheng, Y. and Howard, K. (1997). The *Drosophila* protein Wunen repels migrating germ cells. *Nature* **385**, 64-66.
- Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I. and Littman, D. R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* **393**, 595-599.