Developmental Cell, Vol. 6, 589–596, April, 2004, Copyright ©2004 by Cell Press

Autonomous Modes of Behavior in Primordial Germ Cell Migration

Short Article

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Summary

Zebrafish primordial germ cells (PGCs) are guided toward their targets by the chemokine SDF-1a. PGCs were followed during three phases of their migration: when migrating as individual cells, while remaining in a clustered configuration, and when moving as a cell cluster within the embryo. We found that individually migrating PGCs alternate between migratory and pausing modes. Pausing intervals are characterized by loss of cell polarity and correlate with subsequent changes in the direction of migration. These properties constitute an intrinsic behavior of PGCs, enabling erasure of prior polarity and re-sampling of the environment. Following migration arrest at a site of high SDF-1a levels, PGCs resume migration as a cluster. The seemingly coordinated cluster migration is a result of single-cell movement in response to local variations in SDF-1a distribution. Together, these behavioral modes allow the cells to arrive at specific destinations with high fidelity and remain at their target site.

Introduction

Organ and tissue development and function rely on the cooperation among different cell types. In many cases, cells originate at a distance from the structure they participate in building and migrate directionally to reach that site. Guided-cell migration is involved in pathological conditions as well. For example, chemotactic signals attract macrophages and lymphocytes to areas of chronic inflammation (D'Ambrosio et al., 2003; Lukacs, 2001) and could also lead to a distinct metastatic pattern of certain cancers by attracting tumor cells to specific organs (Murphy, 2001). Elucidating the mechanisms that enable migrating cells to reach their targets, either as individual cells or in a group, and to maintain their position after arriving at the target is therefore a central theme in development and disease. A major group of molecules that functions in guiding cell migration is the chemokine family of secreted molecules and their seven-transmembrane receptors (Horuk, 2001).

An organ whose development in many species relies on directed-cell migration is the gonad. This organ gives rise to gametes and is composed of two major cell populations, somatic and germ cells. Commonly, germ cells (termed primordial germ cells [PGCs] at this stage) are specified in positions that are distinct from the location where the gonad develops and therefore have to migrate toward this site (Starz-Gaiano and Lehmann, 2001; Wylie, 2000). PGC migration has been studied in various organisms leading to the identification of molecules that are important for providing the cells with directional cues (Ara et al., 2003; Doitsidou et al., 2002; Kunwar et al., 2003; Molyneaux et al., 2003; Moore et al., 1998; Van Doren et al., 1998; Zhang et al., 1997).

PGC migration in zebrafish takes place during the first 24 hr of embryonic development (Weidinger et al., 1999, 2002). The migration route of the PGCs is prefigured by the dynamic expression pattern of the chemokine SDF-1a whose receptor, CXCR4b, is expressed by the migrating cells (Doitsidou et al., 2002). Responding to the chemotactic signals provided by SDF-1a, PGCs migrate in distinct steps during which they arrive at intermediate targets before reaching their final target (Weidinger et al., 1999, 2002). In contrast, cells in which CXCR4b/SDF-1a signaling is disrupted migrate nondirectionally and consequently are distributed throughout the embryo (Doitsidou et al., 2002; Supplemental Figure S1 [http://www.developmentalcell.com/cgi/content/full/6/4/589/DC1]).

Considering the general properties of the zebrafish system, PGC migration in this organism is an excellent in vivo model for studying the characteristics of chemokine-directed cell migration during development and disease (Dooley and Zon, 2000; Raz, 2003). To determine the cellular mechanisms governing PGC migration in zebrafish, PGCs were followed during three major phases of their migration: when they migrated as individual cells, while remaining in a clustered configuration at one position, and during the phase at which they migrate as a cluster. During their migration as individual cells, PGCs alternate between migratory and stationary behaviors where the latter is coupled with loss of cell polarity and correlates with changing the direction of migration. After clustering at a position of high SDF-1a levels, PGCs show lack of polarity and active migration. Finally, a detailed analysis of the subsequent stage, when PGCs migrate as a cell cluster, revealed that during this phase too, the movement of the cluster is a consequence of the combined individual cell behavior.

Results and Discussion

PGC Behavior during Migration as Individual Cells

The in vivo analysis of PGCs migrating as single cells is pertinent to diverse individual-cell migration processes such as pioneer axon guidance, migration of neural crest cells, and migration of cancer cells from the primary tumor (Christiansen et al., 2000; Dickson, 2002; Friedl and Wolf, 2003). We initially analyzed cells as they move toward their first intermediate target (Supplemental Figure S1; Doitsidou et al., 2002; Weidinger et al., 1999) by assessing their speed and compared it to that of PGCs in embryos in which SDF-1a signaling was inhibited. We found that wild-type cells migrate at an average speed of 2 μm/min (wt in Figure 1A). Interestingly, in embryos whose SDF-1a signaling was disrupted, cells migrate at

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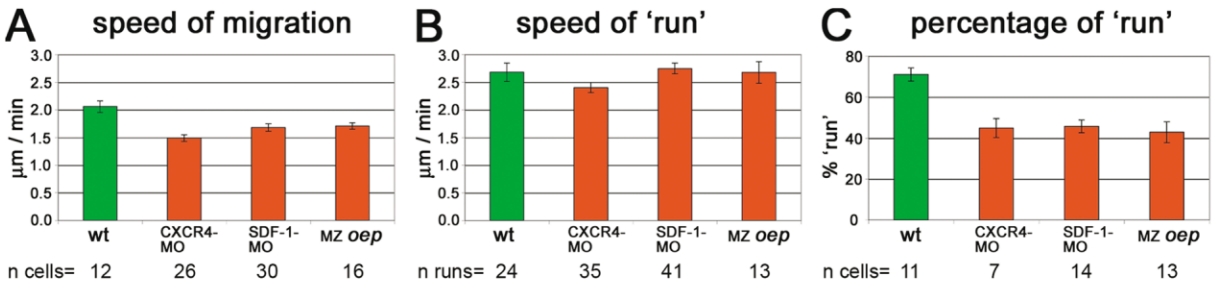
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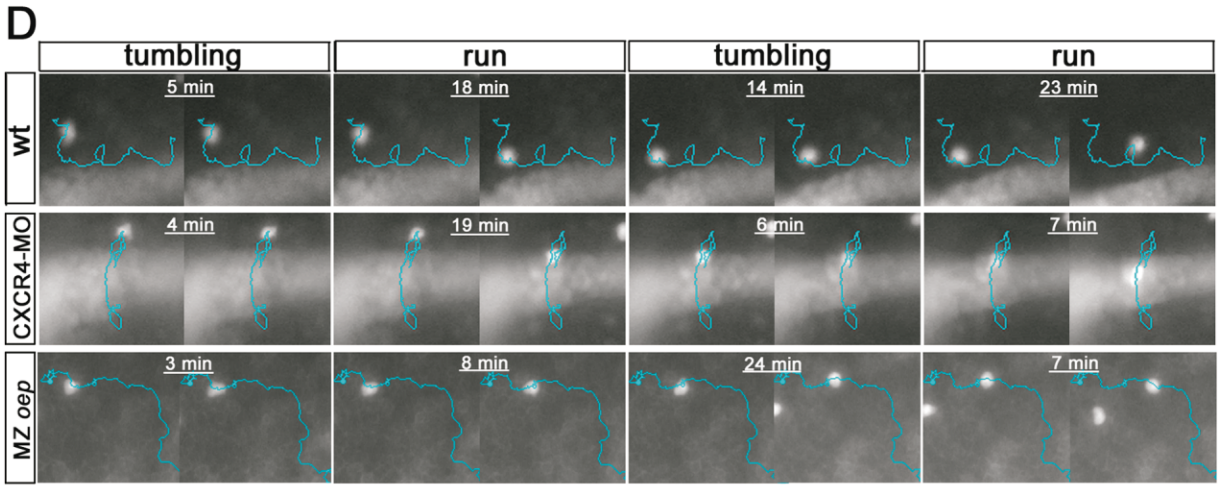
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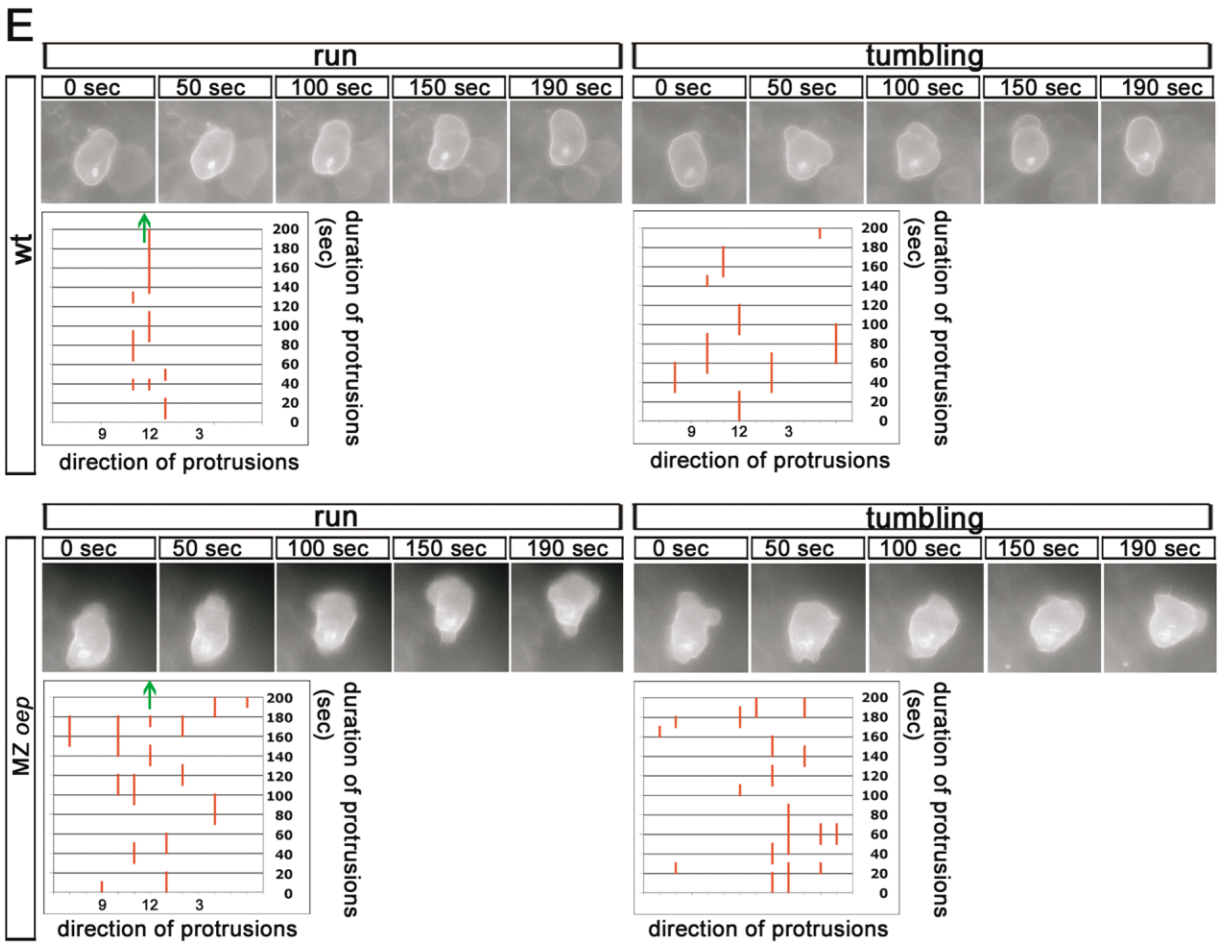
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a reduced speed of 1.7 μm/min or less (Figure 1A, $p < 0.0001$ for CXCR4-MO, $P = 0.0027$ for SDF-1a-MO, and $P = 0.0018$ for MZ *oep*). Disruption of SDF-1a signaling pathway was achieved by employing morpholino (MO) antisense oligonucleotides to inhibit the translation of *sdf*-1a (SDF-1-MO) or *cxcr*4b (CXCR4-MO) (Doitsidou et al., 2002).

To account for the difference in migration speed, we have closely monitored the PGCs as they move on their delineated tracks. We found that migrating PGCs cycle between two behavioral modes; in one they move forward (hereafter referred to as "run") and in the other they stop and remain on the spot (referred to as "tumbling") (Figure 1D). Importantly, these modes of behavior appear to be independent of SDF-1a signaling; when we examined the nature of PGC migration in embryos in which SDF-1a signaling was knocked down, we also identified the two alternating modes (CXCR4-MO in Figure 1D and SDF-1-MO, not shown). The idea that tumbling and run behavior represent an intrinsic property of the migrating cell is most convincingly demonstrated by the analysis of PGC migration in maternal zygotic *one-eyed pinhead* (MZ *oep*) mutant embryos. In these embryos, no expression of the ligand is detected and the germ layers with which the PGCs normally interact, the mesoderm and endoderm, are absent (Supplemental Figure S1A; Gritsman et al., 1999; Weidinger et al., 1999). Despite these dramatic changes, most of the cells are motile and exhibit the characteristic run and tumble behavior (Supplemental Figure S1B and Figure 1D). Hence, these two modes appear to constitute an intrinsic property of PGCs, presumably inherent in their migratory nature.

Considering these findings, the higher speed exhibited by migrating wild-type PGCs relative to their MO-treated or mutant counterparts could stem from either faster migration during run and/or spending more time in run phases. To distinguish between these possibilities, we first measured the speed of PGCs during the run phase in all experimental categories and found that they all show similar values, with slightly lower values for CXCR4-MO (Figure 1B). The most significant difference between wild-type cells and cells in which the SDF-1a signaling was reduced was revealed when the proportion of run *versus* tumbling in a given time frame was assessed. From this analysis, it became apparent that wild-type PGCs spend more time in run phases than do their experimental counterparts (Figure 1C). In addition, the duration of each run was significantly longer in wild-type PGCs (13.5 min ± 1.7 SEM for cells in wild-type embryos compared with 8.6 min ± 0.9 or less in manipulated embryos $p < 0.006$). We therefore conclude that the increased duration of the runs in wild-type cells represents the major contribution to their higher speed during migration.

Throughout their migration, PGCs extend pseudopodia and fillopodia (Weidinger et al., 2002; and data not shown). To characterize the nature of run and tumbling phases of PGC migration in greater detail, we monitored PGCs for the direction of pseudopodial protrusions and their respective duration. Examples of two cells analyzed in this manner, each during run and tumbling phases, are shown in Figure 1E. Examination of wild-type PGCs revealed that during the run phase, cells extend protrusions mainly in the direction of the movement or in its immediate vicinity and assume an elongated appearance. In contrast, during tumbling, PGCs effectively remain on the spot and extend protrusions all around the cell perimeter, thereby losing the polarized appearance. Moreover, while tumbling, PGCs extend multiple, shorter-lived protrusions as opposed to fewer protrusions during run, of which some persist for minutes (Figure 1E and Supplemental Movie S1). This distinctive nature of tumbling appears to be maintained upon knock down of SDF-1a signaling (data not shown) and in the absence of the normal cellular environment (in MZ*oep* mutants, Figure 1E). In some cases, when directional cues or signaling is disrupted, affected PGCs perform run that deviates from that observed in wild-type embryos. Here, run is associated with more protrusions that are more widely spread with respect to the migration vector while the cells keep an elongated morphology (Figure 1E and Supplemental Movie S1). Thus, although cell polarity can be obtained without a polarized signal, this polarity is frequently less pronounced when compared with that achieved by cells responding to asymmetric distribution of the ligand.

In summary, individually migrating PGCs alternate between two behavioral modes independently of the activation status of the directional signaling pathway, or of the cellular environment. However, strictly depending on directional signaling activity, PGCs spend longer times in run and therefore exhibit an overall faster migration.

Figure 1. Migrating PGCs Cycle between Two Behavioral Modes—Run and Tumbling

(A and B) Graphic representation of the mean overall speed of PGC migration and of run phase, in wild-type (wt) embryos, in chemokine-receptor and ligand knockdown embryos (CXCR4-MO and SDF-1-MO, respectively), and in mutants expressing no SDF-1a and lacking mesodermal and endodermal tissues (MZ *oep*).

(C) The proportion of time migrating cells spend in the run phase. For wild-type PGCs, values were obtained from cells migrating toward the first intermediate target (7.5–9.5 hr postfertilization, hpf), and for other experimental categories, values were obtained within the time window of 7.5–12 hpf. In (A)–(C), mean values ± standard error of the mean (SEM) are shown.

(D) Run and tumbling intervals performed by individual PGCs are displayed on their delineated 190 min tracks of migration starting at late gastrulation (7.5–9 hpf). The snapshots from a time-lapse movie represent the beginning and end of two consecutive tumbling and run phases within each track.

(E) Cellular morphology of PGCs during run and tumbling phases. Snapshots from a time-lapse movie of representative single cells (of $n = 10$ for wild-type cells, $n = 5$ for MZ *oep*) performing run and tumbling are shown (see Supplemental Movie S1). The scatter graphs represent analysis of the corresponding cells, where the duration of each extended protrusion is plotted against its direction. The direction of protrusions corresponds to that on a clock, where 12 o'clock signifies the direction of migration during run (green arrow), or the direction of the first extended protrusion during tumbling.

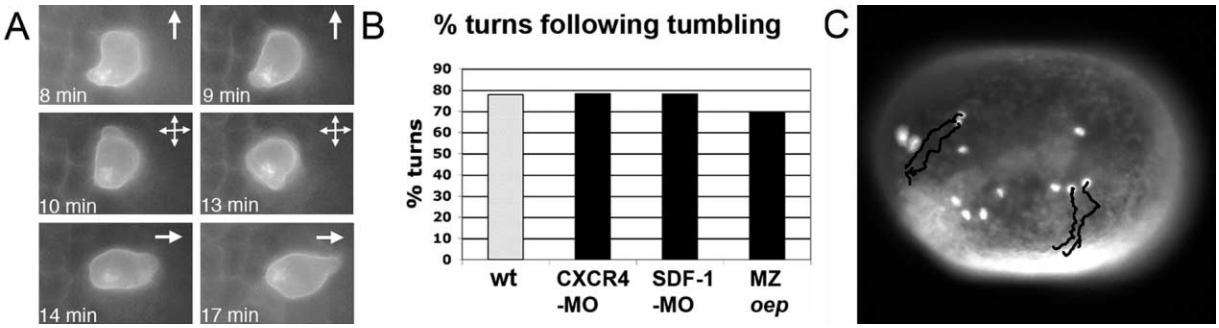


Figure 2. Tumbling Behavior Is Correlated with Alterations in the Direction of Migration

(A) An example of a cell changing the direction of migration (lower panels) with respect to its original direction (upper panels) following tumbling (middle panels).

(B) A graph showing the proportion of turns occurring following tumbling phases (n = 10–23 turns for each experimental category).

(C) A 7.5 hpf wild-type embryo showing two pairs of PGCs and the corresponding paths (black tracks) along which they have migrated in the following 2 hr (see Supplemental Movie S2). The PGCs were selected on the basis of their close proximity within each pair and thus their similar position relative to *sdf*-1a expression domain during the pertinent developmental stage. Anterior is to the left.

Considering that tumbling is an integral part of PGC migration, we sought to determine a function this behavior might be associated with. Interestingly, we noticed that a change of direction is more likely to be carried out by cells of all experimental categories following tumbling (Figures 2A and 2B). A similar behavior has been described for chemotactic bacteria and axon growth cones where a change in the direction of migration is preceded by a tumbling or a pausing phase, respectively (Berg and Brown, 1972; Skaliora et al., 2000). The tumbling phase could therefore facilitate a change in direction by bringing about an erasure of the prior cell polarity. Following tumbling, PGCs could proceed into a run in a new direction that is either random or biased by positional information encoded by the chemokine. To investigate this issue, the direction of cell migration following tumbling phases was determined. PGCs that do not sense SDF-1a (cells in MO-injected or MZ *oep* embryos) exit the tumbling phase in a random direction relative to the direction in which they were previously migrating (n = 113 turns, Supplemental Table S1). In sharp contrast, cells in wild-type nonmanipulated embryos exhibit a strong bias in their post-tumbling direction of migration; only 2 percent of post-tumbling routes were reversed relative to the pre-tumbling course (n = 41 tumbling phases), whereas 60 percent of the post-tumbling routes remained within 45° left or right of their previous direction. We interpret these results, taken together, as an indication that during the tumbling phase the cell polarity is erased, allowing the cell to correct its migration path based on external directional cues. The spatial distribution of SDF-1a at that point in time would therefore dictate the post-tumbling direction assumed by the chemotaxing PGCs. A direction bias by an existing gradient is exemplified by neighboring cells located in a similar position relative to SDF-1a expression domain; remarkably, the migration tracks of the neighboring cells highly resemble one another (two pairs of neighboring cells in Figure 2C and Supplemental Movie S2).

PGC Behavior at a Clustering Position

Starting at early somitogenesis stages (2–3 hr following the stages analyzed above), PGCs form two bilateral clusters at the level of the first three somites (Weidinger et al., 1999). At that time, *sdf*-1a is strongly expressed by a large number of lateral-plate-mesodermal cells at these positions (Doitsidou et al., 2002; Figure 3A, left panel) and it is not until about 3 hr later that clustered PGCs leave this position and resume migration (Weidinger et al., 1999; and see below). The behavior of PGCs at their target site pertains to mechanisms responsible for maintaining various cell types at a given position. Prominent examples are those in which cells take part in constructing tissues or organs or when they form a secondary tumor in the case of cancer. We sought to understand the basis for PGC persistence at the same position and determine the associated cellular morphology during that stage. Examining the clustered PGCs revealed that the cells extend small protrusions in all directions and virtually remain on the spot (Figure 3A, Supplemental Movie S3). Given the stable and restricted expression pattern of *sdf*-1a where PGCs reside during this time window (Doitsidou et al., 2002), we hypothesized that the peak level of SDF-1a at this position leads to a uniform stimulation of the receptor and retention of the cells there. If so, subjecting PGCs to a local high concentration of SDF-1a should result in a cellular phenotype similar to that of PGCs in a cluster. To reproduce such conditions experimentally, the endogenous SDF-1a activity was knocked down and *sdf*-1a and activated TARAM-A RNAs were coinjected into one of 16 blastomeres, thereby generating a clone of endodermal cells expressing SDF-1a (Peyrieras et al., 1998; see Experimental Procedures). The morphology and behavior of the cells attracted to the ectopic source of SDF-1a were then examined. Significantly, we found that PGCs can form clusters at positions of ectopic SDF-1a expression (Supplemental Figure S2 and Supplemental Movie S4). In agreement with our hypothesis, PGCs found on such clonally marked SDF-1a-expressing cells (left image in Figure 3B) presented similar characteristics to those of clustered PGCs; the cells extended protrusions in all directions and barely moved (Figure 3B and Supplemental Movie S3). Scatter graph analysis plotting the duration of successive protrusions against their direction clearly illustrates the highly comparable patterns of PGC

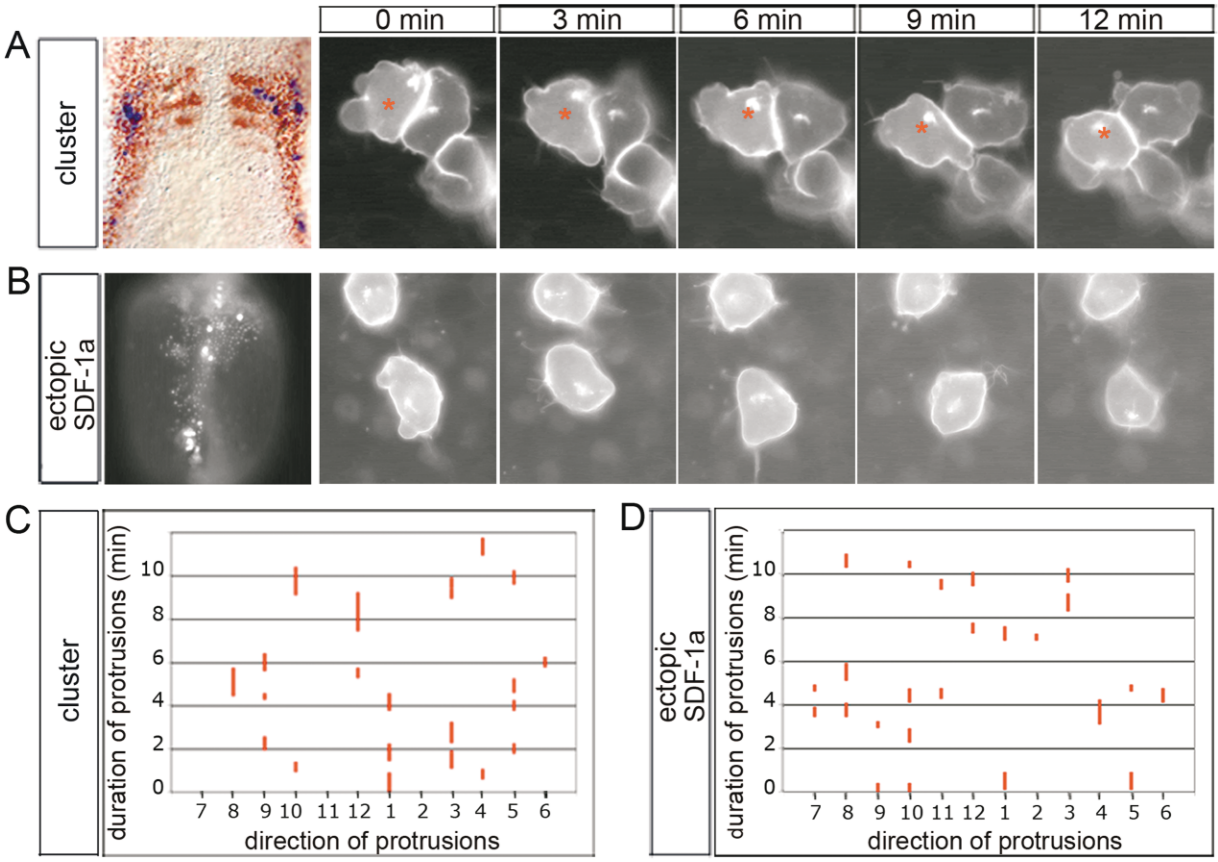


Figure 3. Behavior of PGCs in a Cluster

(A) Image on the left: in situ hybridization of a wild-type embryo at the 4-somite stage probed with *nanos*-1 to visualized PGCs (blue) and with *sdf*-1a (brown). Anterior is up. Shown in addition in (A) are snapshots derived from a time-lapse movie in which clustered PGCs were recorded in a 5-somite wild-type embryo. A single nonmotile cell is marked in red.

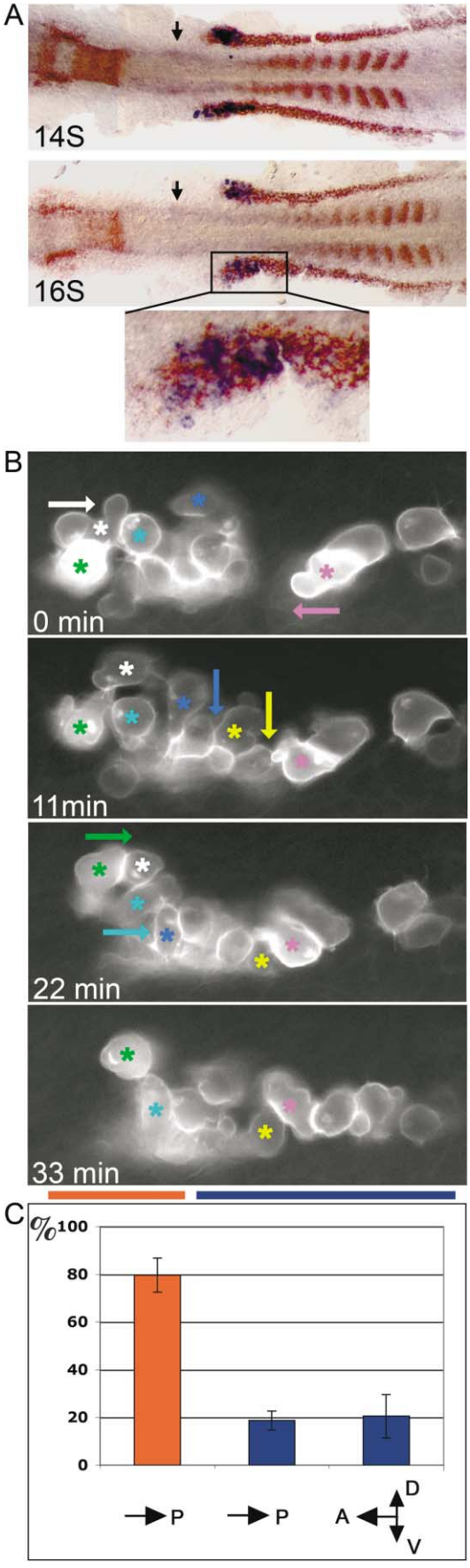
(B) Image on the left: embryo at a 4-somite stage showing GFP-labeled PGCs found on endodermal cells whose nuclei are marked. The rest of (B) presents snapshots from a time-lapse movie showing one such PGC that was experimentally subjected to a localized stable SDF-1a signal expressed by the endodermal clone.

(C and D) Graphical presentation of the cellular morphology of a single PGC (of n = 9 cells) in the cluster (marked in red in [A]) and a representative PGC (n = 5) positioned in an experimentally generated field of SDF-1a-expressing cells (lower cell shown in [B]). In each scattered graph, the duration of a given protrusion is plotted against its direction as in Figure 1.

behavior in wild-type and experimental embryos (Figures 3C and 3D, respectively). A similar cell behavior was observed in an independent experiment in which SDF-1a was produced by the PGCs themselves, thus generating local high concentrations of the chemokine around each cell (data not shown). In conclusion, loss of cell polarity in stable peak concentrations of the chemokine results in a migratory arrest. Such a strategy should allow for bringing cells together and keeping them at the site where they make up tissues and organs.

PGC Behavior during Migration as a Cluster of Cells

About 3 hr after their formation, the PGC clusters migrate to a more posterior position within the embryo toward the site where the gonad develops (Knaut et al., 2002; Weidinger et al., 1999, 2002). Migration of cell clusters is a common phenomenon in development (e.g., Montell, 2003; Ribeiro et al., 2003) and disease (Friedl and Wolf, 2003), and in the case of zebrafish PGCs, it is thought to be directed by SDF-1a (Doitsidou et al., 2002). During this step, PGCs migrate as a cluster, following the shifting anterior front of *sdf*-1a expression domain (Figure 4A). It is possible that clustered PGCs migrate coordinately depending on cell-cell interaction for relaying the SDF-1a directional cues. An example for this mode of migration is that of tracheal cells in *Drosophila* where the cells remain firmly connected with each other throughout the migration process, which is directed by cells at the tip of the branches (Ribeiro et al., 2002; Sato and Kornberg, 2002). Similarly, the migration of border cells during *Drosophila* oogenesis is thought to be guided and powered by a long cellular extension that is formed by one of the cells in the migrating cluster (Fulga and Rørth, 2002). If similar mechanisms were responsible for the posterior migration of the PGC cluster, it would be expected that the cells would exhibit a uniform coordinated movement. Alternatively, the posterior migration of the cluster could reflect the sum of individual cell behaviors, each responding independently to a local concentration of SDF-1a. A prediction



of the latter supposition would be the independent migration of PGCs as single cells within a cluster. Observing PGCs at the relevant stages, we found that the anterior cells in the cluster, the cells overlapping with the moving front of the *sdf*-1a expression domain, are primarily those that migrate in a posterior direction. Specifically, 80% of the cells that assumed the most anterior position in the cluster at any time during migration moved posteriorly, as opposed to only 19% of cells located elsewhere in the cluster (n = 55 cells from 11 clusters; Figures 4B and 4C and Supplemental Movie S5). In summary, no coordinated migration is apparent among clustered PGCs as they move posteriorly and no stable close cell-cell interactions are observed. Rather, cells move independently from one another and unless found in the most anterior position in the cluster, they show a short-range migration in any direction. Anterior PGCs on the other hand, show a preference for posteriorly directed migration, in response to local changes in the distribution of the chemokine. As these cells migrate through the cluster posteriorly, the cells that have now become anterior will be those to follow the dynamically moving high point of SDF-1a. Such continuous waves of anterior cells moving in posterior direction culminate in arrival of clustered PGCs at the site of the future gonad. This description of the PGC cluster migration together with the dynamics of *sdf*-1a expression (Doitsidou et al., 2002) rules out the hypothesis postulating that the final target functions as a distinct attraction center acting on the cells from a distance (Weidinger et al., 2002).

Conclusions

Studying zebrafish primordial germ cell migration in vivo, we identified several behavioral modes that are likely to be relevant for other migratory cells, as well, in particular those guided by chemokines. We find that cell polarity and motility are gained in the absence of any apparent extrinsic asymmetric signal and independent of the cellular context. This behavior is strikingly different from that described for neutrophils in which the

Figure 4. Migration of the PGC Cluster toward the Region Where the Gonad Develops Is the Culmination of Noncoordinated Migration of Individual Cells

(A) Wild-type embryos at 14- and 16-somite stages showing posterior movement of the PGC cluster (*nanos*-1 expressing blue cells) along with *sdf*-1a expression pattern (brown). Anterior is to the left and the position of the first somite is indicated by an arrow. The inset shows a high-resolution image of clustered PGCs whose anterior cells overlap with the anterior border of *sdf*-1a-expression domain.

(B) Images from a time-lapse movie of clustered PGCs migrating toward their target in a 13-hr-old embryo. Individual cells are marked by an asterisk and an arrow of the same color signifying the direction of migration of that cell (Supplemental Movie S5).

(C) A graph presenting the percentage of clustered PGCs that move in a given direction. Mean values ± SEM are shown. P signifies posterior; A, anterior; D, dorsal; and V, ventral. The red color denotes anterior PGC of the cluster and blue denotes the rest of the cells in the cluster. The position of cells defined as anterior cluster cells (red bar) and the rest of the cluster cells (blue bar) are shown below (B).

chemotactic signal is crucial also for attaining cell polarity and motility, but is similar to that described for *Dictyostelium discoideum* (reviewed in Devreotes and Janetopoulos, 2003).

Interestingly, when encountering polar directional cues, as is the case prior to arrival at sites that constitute intermediate targets, PGCs do not perform continuous migration. Rather, the migration is interrupted by phases characterized by a loss of morphological polarity that is often followed by changes or corrections in the direction of migration. Although this description of cellular behavior is reminiscent of that of bacterial chemotaxis (Berg and Brown, 1972), important differences between the systems should be noted. First, while the exit from the tumbling phase in bacteria is not biased by the attractant, following tumbling, PGCs repolarize and migrate in the same general direction they were traveling before, most probably in response to directional cues provided by SDF-1a. Second, in contrast to bacteria, which effectively adapt and continue to exhibit a run behavior at high uniform concentrations of the attractant, PGCs that arrive at their target lose their polarity and stop migrating. This mode of migration allows precise, effective, and stable organization of cells in their target tissue, which is a pivotal feature of organogenesis. Monitoring the concurrent alterations in the organization of the cytoskeleton and the effect of regulators of actin and tubulin polymerization on PGC migration should help deciphering the molecular basis for these modes of cellular behavior.

Finally, our analysis of the migration of the PGC cluster underscores the dominant role of single motile-cell behavior even when moving in a group. The apparent lack of coordinated cell behavior and the absence of stable cell-cell interaction within the group may contribute to augmenting cell metastasis, particularly in cases where a role for CXCR4 has been suggested (e.g., Muller et al., 2001; Staller et al., 2003). Nevertheless, this type of migration provides a simple and flexible mode of transporting groups of cells in the developing embryo; important parameters such as the cluster shape and velocity are controlled by a single factor, namely, the dynamic changes in the expression pattern of the chemokine SDF-1a dictated by the promoter activity of this gene. Determining the precise spatial distribution of the SDF-1a protein with respect to the migrating cells in live embryos should provide a more refined view of the direct cellular response to the dynamic changes in the expression pattern of this chemokine.

Experimental Procedures

Whole-Mount In Situ Hybridization

In situ hybridization was performed as previously described (Jowett and Lettice, 1994) with modification according to Hauptmann and Gerster (1994) and Weidinger et al. (2002). To visualize PGCs, *nanos*-1 (Köprunner et al., 2001) was used as a probe and *sdf*-1a expression pattern was visualized with an *sdf*-1a probe (Doitsidou et al., 2002).

Time-Lapse Analysis and Tracking of PGC Migration Routes and Speed

For time-lapse movie analysis, PGCs were labeled by microinjecting one-cell stage embryos with 150 pg *gfp-nanos*-1 RNA or farnesylated-*egfp-nanos*-1 RNA (Köprunner et al., 2001; Weidinger et al., 2002). To achieve faint membrane labeling of the surrounding somatic cells, 4 pg of farnesylated-*egfp-globin* RNA (Weidinger et al., 2002) was coinjected. Movies and PGC tracks and speed were obtained using MetaMorph software (Universal Imaging). Relative proportions of run, tumbling, and turns performed by PGCs were calculated by analyzing migrating cells traveling on their delineated tracks. The speed of migrating PGCs was corrected for the surrounding morphogenetic movements.

Fish Strains and Experimental Manipulations

As wild-type, zebrafish (*Danio rerio*) of the AB genetic background and MZ *oep*^{tz257} or MZ *oep*^{m134} mutant embryos (Gritsman et al., 1999) were used. For knockdown experiments, 0.4 pmole of morpholino oligonucleotide directed against CXCR4b and SDF-1a (R4b-2-MO and SDF-1a-2-MO, respectively; Doitsidou et al., 2002) was injected into one-cell stage embryos.

For ectopic expression of SDF-1a in the endoderm, endogenous SDF-1a was knocked down by SDF-1a-2-MO. 1 pg of activated TARAM-A that drives blastomeres toward an endodermal fate (Peyrieras et al., 1998) was coninjected with 20 pg of morpholino-resistant *sdf*-1a RNA (Doitsidou et al., 2002) and with 8 pg of *ecfp-h1m-globin3′*UTR (serving as a nuclear clonal marker; Müller et al., 2002) into a marginal blastomere of 16-cell stage embryos.

Acknowledgments

We dedicate this work to the memory of Anat Krauskopf. We are grateful to M. Doitsidou, K. Dumstrei, and K. Slanchev for critical comments on the manuscript. We thank M. Doitsidou for helpful discussions, B. Raschke and J. Doerries for technical assistance, and C.-P. Heisenberg for fish mutant strains. This work was supported by the grants from the DFG and the VW Stiftung to E.R.

Received: December 19, 2003
Revised: February 6, 2004
Accepted: February 10, 2004
Published: April 12, 2004

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