Molecular Mechanisms Governing Primordial Germ Cell Migration In Zebrafish

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Molecular Mechanisms Governing Primordial Germ Cell Migration In Zebrafish

Moleculaire mechanismen die de migratie van primordiale geslachtscellen in zebravis controleren

Maria Doitsidou

Proefschrift

Ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof.dr.ir. L. Speelman,
in het openbaar te verdedigen
op maandag 9 mei 2005
des namiddags te vier uur in de Aula.

ISN: 7460568

ISBN: 90-8504-191-3

Index

Summary

In most sexually reproducing organisms primordial germ cells (PGCs) are specified early in development in places that are distinct from the region where the somatic part of the gonad develops. From their places of specification they have to migrate towards the site where they associate with somatic gonadal cells and differentiate to the gametes. The question concerning the molecular mechanisms that guide PGCs during their migration and allow them to reach their target is the focus of this work. The process was investigated in zebrafish, where the extrauterine development of the embryos their translucency allows monitoring cell migration at high resolution.

Previous studies showed that zebrafish PGCs are specified in four different positions. From these positions the cells perform distinct migration steps until they arrive at their target by the end of the first day of development. During their migration the cells are guided by cues provided by somatic tissues.

To identify the actual molecules that function as the guidance cues, a large-scale antisense-oligonucleotide-based screen was carried out. In this screen, a chemokine receptor, CXCR4b and its ligand, the chemokine SDF1a, were identified as proteins required for guided PGC migration. This pair of molecules had previously been shown to guide cell migration in other model organisms in a variety of developmental processes and disease. For example, SDF-1/CXCR4 signalling guides leukocytes to the sites of inflammation or metastatic tumour cells to sites where they form secondary tumours.

We found that in zebrafish embryos, the receptor CXCR4b is expressed in the migrating PGCs and its ligand, SDF1a, is expressed in the tissues along which the PGCs migrate. Knocking down either CXCR4b or SDF1a impairs PGC directed migration, which becomes evident by the inability of the cells to reach their target. Furthermore, when SDF1a was expressed in ectopic sites in the embryos, PGCs arrived at these sites thus demonstrating the instructive role of this chemokine inPGC migration. Together, these results strongly suggest that SDF1a provides the directional cue for PGC migration in zebrafish. These findings have since been generalized to mouse and chicken, where it was shown that CXCR4 and SDF-1 play an essential role in PGC migration as well.

Interestingly, in *Drosophila* a different biochemical pathway was shown to be important for providing directional cues for migrating PGCs, namely, the cholesterol/isoprenoid biosynthesis pathway. To determine whether this pathway plays a similar role in zebrafish, a 'block and rescue' pharmacogenetic approach was employed. Small chemical compounds were utilized to inhibit distinct steps in the cholesterol and isoprenoid synthesis pathway and the effect on PGC migration was examined. Using this approach, we showed that blocking HMGCoA reductase (an enzyme that catalyses the rate limiting step in cholesterol synthesis) results in slower PGC migration. As a consequence, PGCs in treated embryos were frequently found in abnormal locations. We then determined which components of the cholesterol/isoprenoid biosynthetic pathway that act downstream of HMGCoAR are involved in this process. We could demonstrate that Geranylgeranyl transferase 1 (GGT1) activity in the isoprenoid branch of the pathway is important for optimal PGC motility.

Chapter 1

General Introduction

General Introduction

CELL MIGRATION

Cell migration is an essential process that plays a central role in a wide range of biological phenomena throughout the animal kingdom. In embryogenesis cell migration is the common theme underlying morphogenesis (Gilbert, 2000). For example, during gastrulation large groups of cells migrate to form the three germ layers of the embryo. Later in development, different cell types migrate from various parts of the embryo towards specific locations where they become organized and differentiate to form different tissues and organs. Analogous cell migration processes continue to occur in the adult organism where they are important for homeostasis, maintaining tissue integrity (e.g., in wound healing) and for immune responses.

The failure of cells to migrate or conversely their migration to inappropriate positions can lead to pathological consequences. Pathological conditions that result from aberrant cell migration can be manifested as vascular diseases, osteoporosis, chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis, metastatic cancer, and mental retardation (Cotran et al., 1999). Thus, investigating the fundamental mechanisms underlying cell migration can lead to a better understanding of development and physiology and also holds the promise of effective therapeutic approaches for treating disease.

Two distinct ways for guiding migrating cells have been described: In the case of chemotaxis, motile cells are guided by gradients of secreted signalling molecules, which are produced in distinct locations and act at a distance to attract migrating cells towards the source or repel them away from it (reviewed by(Arkowitz, 1999; Firtel and Chung, 2000)). Well-established examples of chemotaxis during embryonic development include the development of primary branches in the *Drosophila* tracheal (respiratory) system and the migration of the *C. elegans* sex myoblast cell towards the gonad. In both cases, the migration of these cells is regulated by the secretion of the fibroblast growth factor (FGF) (for reviews(Blelloch et al., 1999; Forbes and Lehmann, 1999)). A different way of guiding migrating cells is by providing directional information through the distribution of extracellular matrix (ECM)) components (e.g. substrates for adhesion molecules) that favour migration in a certain direction. This phenomenon of contact guidance is considered to play an important role in the migration of neural crest cells (reviewed in (Perris and Perissinotto, 2000)).

The mechanisms of cell migration have been studied primarily in cell culture, which permits the analysis of cell behaviour in a controlled environment. Analysis of cell migration *in vivo* sheds light on the mechanisms governing this process in the

context of whole organisms. Some of the major questions concerning cell migration include the determination of the molecular basis of cell motility, the identification of guidance cues and the mechanisms that translate those signals into cell polarization and directed movement.

The work described in this thesis deals with some of the above questions using zebrafish primordial germ cells as an *in vivo* model system to study directed cell migration in the context of a developing organism.

PRIMORDIAL GERM CELLS

All sexually reproducing organisms arise from gametes, which are highly specialized cells that initiate the development of a new organism in the next generation. The gametes, in turn, arise from primordial germ cells (PGCs), a small population of cells that in most animal species is set aside from other cell lineages early in embryonic development and follow a unique differentiation pathway.

Several distinct aspects of primordial germ cell development make this cell population an appealing research topic in several model organisms. Specifically, PGCs show a unique control over RNA transcription, exhibit a unique cell cycle program and show a characteristic cellular and subcellular morphology, properties that distinguish them from neighbouring somatic cells. Furthermore, PGCs perform long-range migration, during which they encounter different cellular environments. Yet, considering that the gametes that PGCs will form should be able to give rise to any cell type of the embryo, PGCs ignore differentiation signals that could potentially interfere with this feature.

Primordial Germ Cell specification

In zebrafish, like in other model organisms such as *Drosophila, C. elegans* and *Xenopus*, primordial germ cells are specified by inheritance of asymmetrically localized cytoplasmic determinants –the germ plasm (Hashimoto et al., 2004; Knaut et al., 2000; Yoon et al., 1997). The germ plasm is characterized by the presence of electron dense structures (the polar granules) that are associated with mitochondria and contain RNA and protein molecules. The specification of PGCs in mammals follows a different strategy. Here, PGC fate is induced during gastrulation through cellular interactions rather than by inheritance of cytoplasmic determinants (reviewed in McLaren, 1999; Wylie, 1999).

The study of PGC development in zebrafish was revolutionized by the cloning of molecular markers that arespecifically expressed in the germline. The first PGC specific marker identified in zebrafish was vasa RNA (Yoon et al., 1997). Vasa encodes an ATP-dependent RNA helicase of the DEAD BOX family that was originally identified in *Drosophila* (Hay et al., 1988; Lasko and Ashburner, 1988). As in several other organisms, the zebrafish vasa RNA is maternally provided and is expressed in the germline throughout development (Braat et al., 1999; Knaut et al., 2000; Weidinger et al., 1999; Yoon et al., 1997). The identification of this marker thus

allowed for the first time a close examination of germ cell development in zebrafish from the earliest embryonic stages to the later stages when the cells differentiate into gametes. Whole-mount in situ hybridization showed that vasa RNA is enriched in four stripes along the four first cleavage planes at the four-cell stage. During cleavage stages, the division of vasa RNA containing cells (termed presumptive PGCs or pPGCs at this stage) is asymmetric, so that only one of the two dividing blastomeres inherits the germ plasm. Following this phase, the germ plasm is distributed uniformly in the cytoplasm, and during cell division it is inherited by both daughter cells.

It is important to note that germ plasm material in zebrafish can initially be detected outside the four main positions, but this material that includes RNA molecules such as vasa and nanos-1 (another germline specific marker (Köprunner et al., 2001)), is gradually degraded in the somatic cells. It was shown that degradation of vasa and nanos-1 RNA is dependent on cis-acting elements found in their 3' untranslated region (Köprunner et al., 2001; Wolke et al., 2002).These elements are sufficient to direct RNA degradation and translational inhibition in the soma, while promoting its stability in the PGCs. These findings proved to be of significant practical importance because it became possible to express proteins of interest specifically in the PGCs by introducing the corresponding RNA fused to these cis-acting elements into early embryos.

Primordial Germ Cell Migration

In most sexually reproducing organisms PGCs are specified early in development in places that are distinct from the region where the somatic part of the gonad develops. Therefore, these cells have to migrate from the position where they are specified to the site where they associate with somatic gonadal cells and differentiate to the gametes.

Studies preceding the one described in this work supported the idea that PGCs in *Drosophila*, chick and mouse are guided by molecular cues provided by somatic cells along their migration route (see below). However, the percise molecular mechanisms responsible for providing PGCs with directional information were largely unknown.

Drosophila

Drosophila PGCs are formed at the posterior pole of the zygote. During gastrulation they are passively carried with the invaginating posterior midgut primordium to the interior of the embryo. From there, PGCs actively migrate across the gut epithelium and then dorsally along its basal site. Subsequently, the PGCs migrate towards the adjacent mesoderm where they associate with somatic gonadal precursor cells, with which they coalesce to form the gonad (Callaini et al., 1995; Jaglarz and Howard, 1994; Jaglarz and Howard, 1995).

Analysis of mutant embryos in which the differentiation of certain somatic tissues was inhibited showed that PGCs are guided by signals produced in their environment. Although several genes are known to be important for proper specification of somatic target tissues and therefore are indirectly required for PGC migration, only four genes have thus far been identified that are specifically involved in

directing PGC migration. Nevertheless, none of these genes encodes a signalling molecule that could directly guide the PGCs, but rather they appear to be required for the production or reception of such signals.

Two of the genes that were identified, the wunen and wunen-2 genes are expressed in regions of the posterior midgut that *Drosophila* PGCs normally avoid (Fig. 1) (Starz-Gaiano et al., 2001; Zhang et al., 1997). When both genes are mutated, PGCs spread out over the surface of the gut and most of them do not reach the gonadal mesoderm. Moreover, when either one of the wunen genes is ectopically expressed in the mesoderm, PGCs avoid this otherwise attractive tissue. Thus, these genes act redundantly to mediate repulsion of the PGCs from specific regions of the gut. *wunen* and *wunen*-2 are homologs of the mammalian lipid phosphate phosphatase-1 (LPP-1) genes, which are transmembrane proteins with an extracellularly oriented catalytic domain (for a review see Sciorra and Morris, 2002). In addition, repulsion of the PGCs by WUNEN2 requires the catalytic domain of the enzyme (Starz-Gaiano et al., 2001). This suggests that the WUNEN proteins act on the surface of the gut epithelium either to produce a repellent signal or to destroy a phospholipid that acts as an attractant.

In contrast to WUNEN, the *Drosophila* 3-hydroxy-3methylglutaryl coenzyme A reductase (HMGCoAR, encoded by the gene *columbus*) is responsible for generating an attractive signal in the target tissue (Fig. 1)(Van Doren et al., 1998). It is expressed in the gonadal mesoderm at the time the PGCs leave the gut and migrate towards this tissue. In the absence of its activity, most PGCs fail to reach the gut and to associate with the somatic gonadal precursors. Conversely, when HMGCoAR is over-expressed in tissues that normally do not express it, PGCs can be found in these ectopic locations. The molecular nature of HMGCoAR indicates that this enzyme is not an attractant *per se* but rather plays a role in the production or modification of the actual attractant. In mammals, HMGCoAR catalyzes the rate-limiting step of cholesterol synthesis. Flies however do not synthesize cholesterol but rather obtain it by ingestion. Consistently, several of the downstream enzymes required for cholesterol biosynthesis are not found in the fly genome (Santos and Lehmann, 2004b). A possible function of HMGCoAR in this context could thus be the production of downstream components, such as lipids, steroid precursors, and isoprenoids, which are utilized in the covalent modifications of proteins some of which could be directly involved in PGC guidance.

Recently, a novel G-protein coupled receptor, trapped in endoderm-1 (tre-1) was identified. This protein is required in the germ cells for the execution of the step when they migrate trough the gut epithelium (Fig. 1)(Kunwar et al., 2003). In embryos from tre-1 mutant females, most germ cells are trapped inside the epithelium. The precise role of Tre-1 in transepithelial migration is currently unknown. Tre-1 could act at short range to mediate interaction between germ cells and the midgut to allow transepithelial migration or it could act as a receptor for a long-range chemoattractant that guides germ cells through the epithelium.

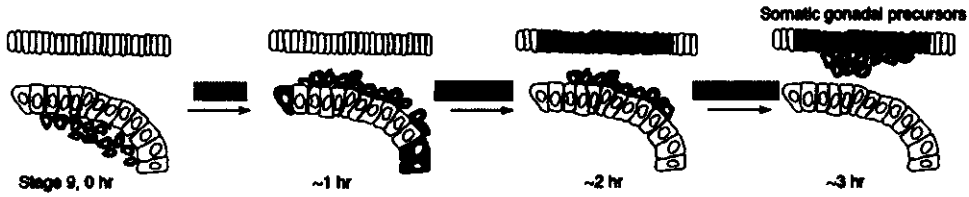

Fig. 1: Expression of trapped in endoderm-1 (tre-1, blue) in the PGCs of Drosophila is required for transepithelial migration out of the posterior midgut. Once out of the posterior midgut, the PGCs are directed towards the mesoderm by the repulsive activity of Wunen and Wunen-2, which are expressed in certain areas of the posterior midgut (red). The PGCs are then attracted to the somatic gonadal precursor cells that express hmgcr (green). (From (Raz, 2004)).

Chick

In the chick embryo, germ cells are specified in the epiblast in the central zone of the embryo proper. From this position, the cells are translocated to the anterior extraembryonic region where they incorporate into the forming vascular network and start to circulate within the blood stream (Eyal-Giladi, 1997; Ginsburg and Eyal-Giladi, 1987; Tsunekawa et al., 2000). Later in development, germ cells leave the vascular system and migrate into the region of where the gonad develops (Fig. 2) (reviewed by (Niewkoop and Sutasurya, 1979)).

Although the mechanism responsible for the extravagation of the PGCs from the blood stream at a position adjacent to the developing gonad was not known, the following embryological experiments suggested that chemotaxis most likely plays a role: First, chick PGCs, isolated from the blood and cultured between gonadal rudiments and other embryonic tissues migrate to the gonadal rudiments (Kuwana et al., 1986). Second, genital ridges from quail embryos grafted behind the optic vesicle of chicken embryos became colonized by chicken germ cells (Kuwana and Rogulska, 1999). Last, the gonads of chick embryos were attractive for mouse PGCs (Rogulska et al., 1971). Thus the chicken gonad appeared to be able to actively attract circulating PGCs and this property appears to utilize factors that are conserved across species.

Recently, following the identification of the molecule guiding PGC migration in zebrafish (the study described in chapter 2 of this thesis), it was demonstrated that the

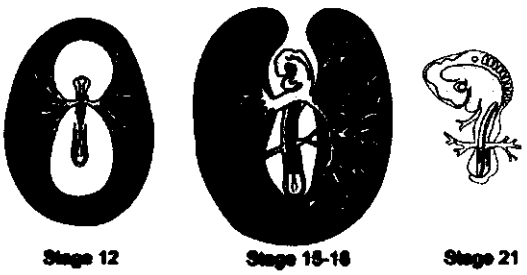

Fig. 2: In chick embryos PGCs use the vascular system as a vehicle to transport them to the region of the gonad.

same molecule (SDF-1) functions in attracting chick PGCs to their target (Stebler et al., 2004). This point will be discussed in chapter 4.

Mouse

In the mouse embryo, primordial germ cells migrate during gastrulation in the region of the posterior primitive streak. From this region they enter the embryonic endoderm by the way of the allantois (Anderson et al., 2000). After the endoderm invaginates to form the hindgut, PGCs are found along the ventral side of the forming organ. Leaving this site, the PGCs migrate actively through the dorsal aspect of the gut and then through the gut epithelium towards the genital ridge (Fig. 3) (Molyneaux et al., 2001).

Several studies highlighted the importance of PGC interactions with the extracellular matrix during their migration.(Anderson et al., 1999; Bendel-Stenzel et al., 2000; Di Carlo and De Felici, 2000; Garcia-Castro et al., 1997). For example, mouse PGCs can adhere to the extracellular matrix (ECM) glycoproteins such as fibronectin and laminim and germ cells lacking the integrin receptor for extracellular matrix proteins cannot migrate to the gonad (Anderson et al., 1999).

Whereas interactions with the ECM (contact guidance) might play an important role in the process of mouse PGC migration, it is considered more likely that directionality is provided by a gradient of a secreted molecule (chemoattraction). Evidence for this notion was provided by *in vitro* experiments in which cultured PGCs were shown to be attracted by gonad primordia explants (Godin et al., 1990). This chemoattractive effect of the gonad primordial is abolished by using antibodies that block the growth factor TGFβ1 (Godin and Wylie, 1991). Nevertheless, an expression pattern of the TGFβ1 molecule that would be consistent with a role in directing mouse PGC migration has not been reported and no *in vivo* evidence for such a role has been described.

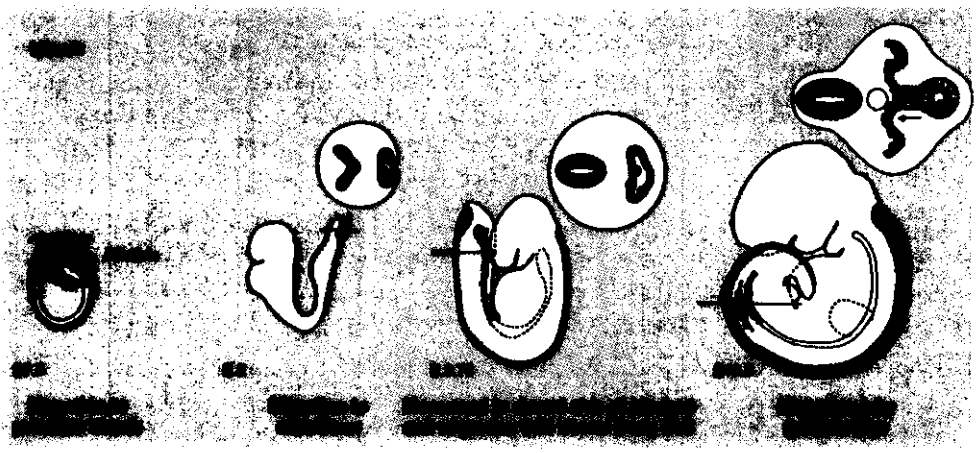

***Fig. 3:** Mouse primordial germ cell migration (lateral views with enlarged cross section next to each stage, line indicates level of section). Embryonic stage 7.5 (E7.5): Germ cells (yellow) moving toward the primitive streak. At stage E8, germ cells are in the endoderm (red); blue, neural fold. At E9.75, germ cells move on the hindgut (red) toward its dorsal side into the lateral body wall. At E10.5, most germ cells have left the hindgut and are in the genital ridge (red and green). (Adapted from (Santos and Lehmann, 2004a))*

Another secreted molecule implicated in PGC migration that -in contrast to TGFβ1- was shown to function *in vivo* is the Steel factor. Steel is expressed along the migration route of PGCs and its receptor, the tyrosin kinase receptor Kit (c-Kit) is expressed in the PGCs. It was suggested that Steel/Kit interaction is required to support migration and survival of the PGCs, but a direct demonstration of the role of Steel as a guidance cue is still lacking (Bernex et al., 1996; De Miguel et al., 2002; Matsui et al., 1990).

As in the case of the chick, following the work described in chapter 2, it was demonstrated that the chemokine SDF-1 plays a role in directing the migrating mouse PGCs towards their target (Ara et al., 2003; Molyneaux et al., 2003). These findings will be discussed in chapter 4 of this thesis.

PRIMORDIAL GERM CELL MIGRATION IN ZEBRAFISH

Zebrafish PGCs are formed in positions that are defined by the first cleavage planes. This arrangement creates an interesting situation where PGCs initiate their migration not from one defined position as described in *Drosophila*, mouse and chicken, but from four different starting points (Fig. 6, A). A further complication is that these four starting points are randomly oriented with respect to dorsoventral axis of the embryo. Thus, the PGCs can be found initially at any position around the margin of the embryo and yet, all of them arrive at their target by the end of the first day of development. The robustness of the mechanisms that guide PGCs in zebrafish became particularly notable when PGCs transplanted into the animal pole of an early embryo – a region that is normally devoid of PGCs and is destined to give rise mainly to head structures- arrived at the region of the gonad (Ciruna et al., 2002).

A first step in determining the mechanisms governing zebrafish PGC migration was a study providing a detailed description of their migration path relative to different somatic structures (Weidinger et al., 1999), (Fig. 4). The migration process could be divided into six distinct steps, culminating in the formation of two cell clusters by the end of the first day of development (Fig. 4, G) (Weidinger et al., 1999)). An important intermediate step during PGC migration is taking place at the end of gastrulation when the PGCs align along the border between the head and the trunk mesoderm (Fig. 4, D). Subsequently, the PGCs migrate towards two lateral positions at the anteroposterior level of the first somite and form two clusters (Fig.4, E). During later somitogenesis stages the PGC clusters migrate posteriorly, to reach their final target at the level of the 8th somite by the end of the first day of development (Fig.4, E-G). The different steps in PGC migration will be discussed again in the next chapter comparing cell position and movement with respect to the expession of the guidance cues that are provided by somatic tissues.

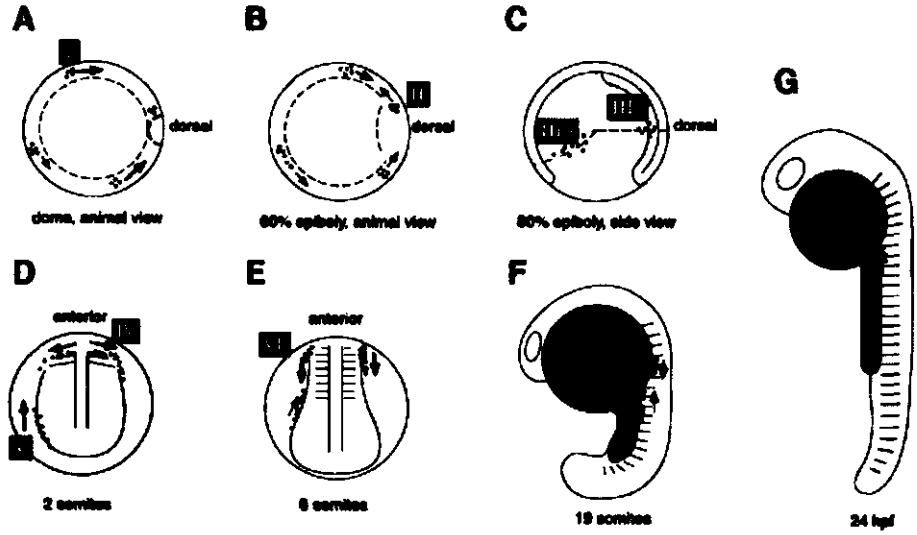

Fig. 4. The six steps of early PGC migration in zebrafish. *Schematic drawings of embryos from dome stage (4.5 hours post fertilization (hpf) to 24 hpf showing the positions and movements of the four PGC clusters. At dome stage, four clusters of PGCs are found close to the blastoderm margin in a symmetrical 'square' shape. All possible orientations of the square relative to the dorsal side of the embryo can be observed.*

(A) Here, an intermediate arrangement is shown with one cluster close to, but not directly at the dorsal side. Before gastrulation, lateral and ventral clusters move towards the dorsal (step I, convergence towards the dorsal).

(B) Clusters located very close to the dorsal migrate away from the dorsal midline and are therefore rarely found on the notochord from the 60% epiboly stage on (step II, exclusion from the dorsal midline).

(C) Dorsally located PGCs align along the border between the head and trunk paraxial mesoderm depicted by a dashed line (step IIIa, alignment along the anterior border of the trunk mesoderm). Ventrally located clusters align at the lateral border of the mesoderm (step IIIb, alignment along the lateral border of the mesoderm).

(D) At the 2-somite stage, most PGCs have arrived in two lines at the level of the first somite. These anterior located PGCs migrate towards the lateral (step IV, formation of two lateral PGC clusters). Cells that were initially located ventrally migrate towards the anterior (step V, anterior migration of trailing PGCs). In this illustration, the positions of the PGCs are drawn relative to the adaxial cells, the somites and the lateral border of the pronephric anlage.

(E) At the 8-somite stage, all anterior PGCs are found lateral to the paraxial mesoderm in a cluster extending from the 1st to the 3rd somite. These clusters start to move towards the posterior (step VI, posterior positioning of the PGC clusters), while the trailing cells continue to migrate anteriorly..

(F) At the 19-somite stage, the main clusters have shifted to more posterior positions and in 60% of embryos some trailing cells are still seen.

(G) At 24 hpf, the PGC clusters are located at the anterior end of the yolk extension, which corresponds to the 8th to 10th somite level. In most embryos, all PGCs have reached this region, only a few trailing cells are found close to the main clusters. (Adapted from (Weidinger et al., 1999))

As discussed in the previous section, studies of PGC migration in other model organisms underscored the importance of somatic tissues along the migrating route in providing the PGCs with directional cues. The description of the migration route of the

PGCs in zebrafish led to similar conclusions. The PGCs appear to align along specific structures of the embryo, they consistently avoid certain tissues and migrate towards defined positions within the developing embryo. Nevertheless, the underlying mechanism responsible for the arrival of the PGCs at their intermediate and final targets was not known.

More direct demonstrations of the control of PGC migration by the somatic environment were obtained when PGC migration was followed in embryos bearing mutations that affect the development of specific structures (Weidinger et al., 1999; Weidinger et al., 2002). This analysis, combined with visualization of GFP-labelled PGCs in living embryos, showed that PGCs respond to positional information provided by somatic tissues and that their absence results in aberrant PGC migration.

A striking example reflecting the importance of guidance cues provided by somatic intermediate targets is the abnormal PGC migration in *spadetail* mutants. The *spadetail* gene encodes a T-box protein required for proper development of the trunk paraxial mesoderm (Griffin et al., 1998). In these mutants, the anterior border of the trunk does not differentiate properly, which is manifested in the absence of specific RNA markers that are normally expressed in this region. As mentioned earlier, the anterior border of the trunk constitutes an intermediate target for the PGCs and indeed in *spadetail* mutants the PGCs do not align in this position. As a result, in many cases PGCs arrive at ectopic positions. Interestingly, one of the ectopic positions in which the PGCs are found in *spadetail* mutants, is a specific region in the head that was considered to serve as an alternative attraction point (Weidinger et al., 1999).

Mutant analysis did not only highlight the importance of the somatic environment in PGC migration, but also gave the first clue regarding the nature of the signals that direct PGC migration. For example, when PGCs migrate from medial places of the embryo to the lateral to form two clusters (see Fig. 4, D-E), they could respond either to repulsive signals emanating from medial somatic tissues or to attractive cues emanating from the target tissue at the lateral (Fig. 5, A). It was possible to distinguish between these options by using mutant strains in which the medial structures that could potentially repel PGCs were genetically eliminated. The result of these experiments showed that when medial structures were missing -for example in the mutant *floating head*, that encodes a homeodomain protein (Talbot et al., 1995)- PGCs were able to leave the medial positions and reach their intermediate target (Fig. 5, B). In contrast, when the position of the intermediate target was altered -for example in *knypek* mutants, that lack the function of a glypican (Topczewski et al., 2001)- PGC migration was changed accordingly (Fig. 5, C) (Weidinger et al., 1999).

The conclusion obtained from similar experiments at different stages of development was that PGCs actively migrate towards their targets, and they do so by responding to attractive cues provided by somatic tissues. Furthermore, based on the analysis of the PGC migration steps, it was possible to draw a map of putative attractive regions in the embryo (shown in Fig. 6). At the onset of the work described in this thesis, the molecular identity of the signals emanating from somatic tissues that are responsible for attracting PGCs was not known.

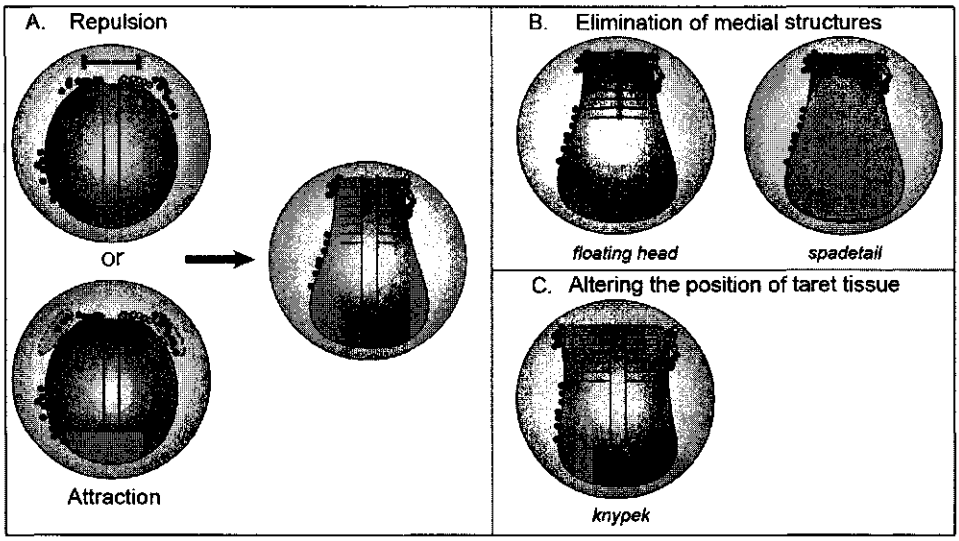

Fig. 5: Directional cues for PGCs.
A. The migration of PGCs away from the dorsal midline could be a response to repulsive signals that are produced by somatic cells in regions vacated by germ cells, or to attractive signals that originate at the targets (dashed line).
B. Schematic representation of two mutants that lack certain medial structures that could putatively repel cells from these locations. The loss these structures had no effect on the ability of the cells to migrate laterally.
C. By contrast, altering the position of the targets by moving them more laterally resulted in a corresponding alteration of the positions towards which the germ cells migrate. Adapted from (Raz, 2003)

Identification of genes important for in guided PGC migration in zebrafish: The aim and the results of this thesis

One strategy to identify genes that are involved in specific developmental processes is to employ genetic screens. Such screens have been successfully used in *Drosophila* and resulted in the identification of genes involved in PGC migration (e.g., the genes *columbus*, *wunen* and *tre*-1 mentioned above). Despite the relative difficulties, genetic screens can also be performed in vertebrates. In particular, zebrafish offers significant advantages for performing such screens, namely reduced costs, extra-uterine development and large numbers of offspring.

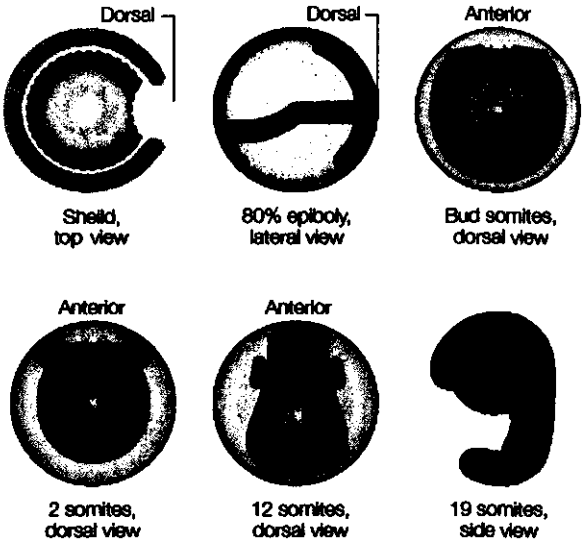

Fig. 6: On the basis of the analysis of germ-cell migration in wild type and mutant embryos, domains that are more attractive for the germ cells are highlighted in yellow (adapted from (Raz, 2003))

In the work described in chapter 2 of this thesis, a genetic screen was employed to identify genes involved in PGC migration (Fig. 7). In this screen genomic information that included the translation start of different genes was used to design modified antisense oligonucleotides (morpholinos) to block the translation of specific mRNAs. The antisense oligonoucleotides were injected into embryos that were subsequently assayed for PGC migration phenotypes. Although the costs involved in such a screen are considerably higher that those of a conventional forward genetic screen, the inherent advantage is the immediate identification of the affected genes.

This large-scale morpholino-based screen resulted in the identification of the chemokine receptor CXCR4b and the chemokine SDF1a as the key guidance factors for PGC migration. This study shows that SDF1a is the molecule that provides PGCs with directional information during all steps of their migration and that the receptor CXCR4b that is expressed by the migrating cells, enables them to respond to the guiding signal.

In chapter 3, the role of HMGCoAR (homologue of the *Drosophila columbus* gene) on PGC migration in zebrafish is examined. As mentioned above, HMGCoAR catalyses the rate limiting step in the cholesterol/isoprenoid biosynthesis pathway and was shown to be important for the generation of attractive cues for *Drosophila* PGCs. This raised the possibility that this molecule plays a similar role in zebrafish. In this chapter, a pharmacogenetic 'block and rescue' approach was employed to define the putative role of HMGCoAR in zebrafish PGC migration and to determine the downstream components of the cholesterol/isoprenoid biosynthetic pathway involved in this process.

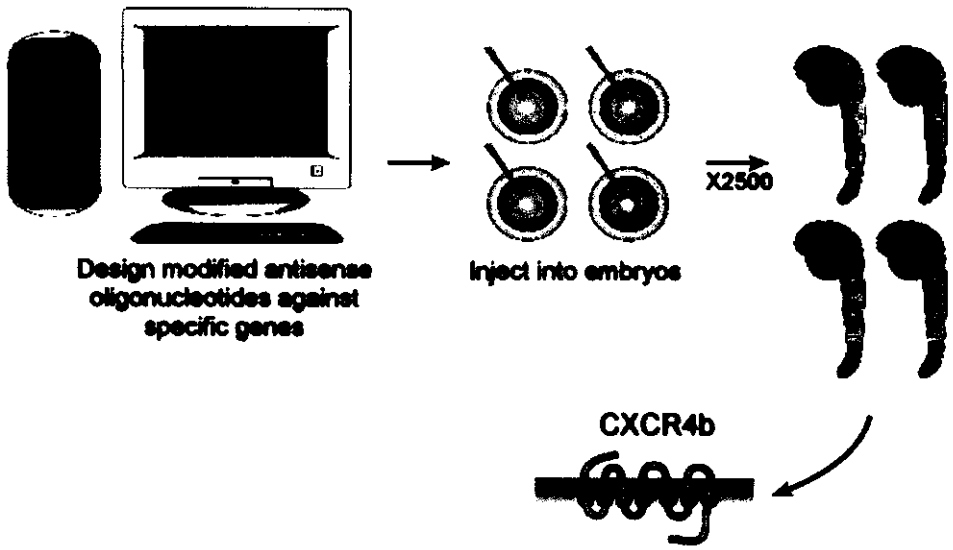

Fig. 7: Identification of the chemokine receptor Cxcr4b by genetic screens for genes that affect germ-cell migration. *Modified antisense oligonucleotide-based screen. Antisense oligonucleotides were designed and injected into embryos. The embryos were assayed at 24 hours post fertilization for abnormal PGC migration. An oligonucleotide that was directed against the cxcr4b gene was found to lead to a severe germ-cell migration phenotype (adapted from (Raz, 2003)).*

Chapter 2

Guidance of Primordial Germ Cell Migration by the Chemokine SDF-1

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This chapter has been published in Cell 111:647-659 (2002)

Guidance of Primordial Germ Cell Migration by the Chemokine SDF-1

ABSTRACT

The signals directing primordial germ cell (PGC) migration in vertebrates are largely unknown. We demonstrate that sdf-1 mRNA is expressed in locations where PGCs are found and toward which they migrate in wild-type as well as in mutant embryos in which PGC migration is abnormal. Knocking down SDF-1 or its receptor CXCR4 results in severe defects in PGC migration. Specifically, PGCs that do not receive the SDF-1 signal exhibit lack of directional movement toward their target and arrive at ectopic positions within the embryo. Finally, we show that the PGCs can be attracted toward an ectopic source of the chemokine, strongly suggesting that this molecule provides a key directional cue for the PGCs.

INTRODUCTION

Directional cell migration during early development and adult life is crucial for the establishment of the embryonic body plan, organogenesis, and organ function. Identification of the molecular cues governing cell migration *in vivo* is of major importance for understanding development and for therapy in cases of diseases resulting from aberrant cell movement. A useful model system for studying the process of directional migration is that of primordial germ cells (PGCs). In most organisms, the formation of a functional gonad depends on the migration of PGCs from their site of specification to the position where the somatic part of the gonad develops (Starz-Gaiano and Lehmann 2001; Wylie 1999 and Wylie 2000). Therefore, the PGCs have to travel long distances within the embryo, which itself undergoes complex processes of morphogenesis and differentiation.

PGC migration has been studied in chick, mouse, *Xenopus*, *Drosophila*, and zebrafish (reviewed in Starz-Gaiano and Lehmann 2001 and Wylie 1999). These studies showed that while migrating, PGCs interact with different somatic structures in the developing embryo. These structures can carry the PGCs along, as part of general morphogenetic movements, repel them from certain regions of the embryo, and attract them toward intermediate and final targets (Deshpande et al. 2001; Godin et al. 1990; Jaglarz and Howard 1995; Kuwana and Rogulska 1999; Starz-Gaiano et al. 2001; van Doren et al. 1998; Weidinger et al. 1999; Weidinger et al. 2002 and Zhang et al. 1997). Yet, despite the fact that this migration process has been studied for several decades in

different model organisms, the molecular nature of the actual signals that direct PGCs toward their intermediate and final targets has remained unknown.

We have chosen to study PGC migration in zebrafish, benefiting from the fast, extrauterine embryonic development, the optical clarity, and the availability of mutant strains and genomic tools. Previously, we have described the migration path of zebrafish PGCs and analyzed the requirement for specific somatic structures in allowing proper migration to take place (Weidinger et al. 1999 and Weidinger et al. 2002). As in other organisms, PGC migration in zebrafish can be divided into several steps that appear to rely on the integrity of specific somatic structures. Nevertheless, even though PGC migration in zebrafish has been described in great detail and the available data support the notion that secreted factors are involved in the process, the identity of such molecules was not known (Braat et al. 1999; Weidinger et al. 1999; Weidinger et al. 2002 and Yoon et al. 1997).

In this work, we have identified the chemokine stromal-cell-derived-factor (SDF)-1a as a pivotal component guiding PGC migration. We show that *sdf*-1a is expressed in domains toward which the PGCs migrate and that alterations in its expression pattern lead to corresponding alterations in the migration route of the cells. Importantly, inhibition of the translation of SDF-1a, or its seven transmembrane G protein-coupled receptor CXCR4b, lead to misguided PGC migration. Taken together, these findings indicate that SDF-1a and its receptor CXCR4b are key molecules directing the PGCs toward their intermediate and final targets.

RESULTS

SDF-1a Is Expressed in Regions toward Which the PGCs Migrate, and Its Receptor Is Expressed in the Migrating PGCs

In the course of a large-scale antisense oligonucleotide-mediated knockdown screen for genes important for PGC migration, we discovered that the seven-transmembrane G protein-coupled receptor CXCR4b (Chong et al., 2001) plays a critical role in the directional migration of PGCs (see below). With significant relevance to our work, it has been previously shown that CXCR4 and its ligand, the chemokine SDF-1, are essential for stem cell homing and mobilization (Peled et al. 1999b and Petit et al. 2002), leuckocyte traffic (Aiuti et al. 1997; Baggiolini 1998; Bleul et al. 1996a and Zou et al. 1998), neuronal cell migration (Zou et al., 1998), nerve growth cone guidance (Xiang et al., 2002), and determination of metastatic destination of tumor cells (Muller et al. 2001 and Murphy 2001). To establish direct relationships between this receptor and PGC migration, we first analyzed the RNA expression patterns of this molecule and its ligand in relation to the position of the PGCs at different steps of their migration.

During early gastrulation stages, at the onset of PGC migration, the cxcr 4b RNA is uniformly distributed within the embryo and is thus expressed also in the migrating PGCs (Figures 1A and 1C). By the end of gastrulation, the RNA is expressed

in the PGCs that align along the lateral plate mesoderm of the trunk and the border between the head and the trunk mesoderm (insert in Figure 1E). During somitogenesis stages, the PGC-specific expression of the receptor is evident in the clustered PGCs (arrowheads and insert in Figure 1G) as well as in cells migrating toward the clusters (small insert in Figure 1G). At 24 hr postfertilization (hpf), the PGCs, found at the anterior part of the yolk extension, strongly express cxcr 4b (arrowhead in Figure 1I). Thus, PGCs express cxcr 4b at the time when they migrate toward their clustering position and continue expressing it after arriving at the site where the gonad will develop.

We next sought to determine whether the CXCR4b ligand expression pattern is consistent with its putative role in PGC migration. CXCR4 has been shown to bind a single molecule, SDF-1, a CXC subfamily chemokine (Baggiolini et al. 1997; Bleul et al. 1996a and Bleul et al. 1996b), and this ligand appears to interact exclusively with the CXCR4 receptor. We therefore cloned the zebrafish *sdf*-1a gene, which encodes a protein that exhibits a high level of conservation with the mouse SDF-1 protein (43% identity at the amino acid level; see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/111/5/647/DC1).

Analysis of the *sdf*-1a spatio-temporal expression pattern revealed a remarkable correlation with the PGC localization at different developmental stages. Specifically, at the onset of gastrulation, *sdf*-1a is expressed around the blastoderm margin with the exception of the dorsal-most aspect of the embryo, coinciding with the position where the PGCs are found at this stage (Figures 1B and 1D; Weidinger et al. 1999 and Yoon et al. 1997). During gastrulation and early somitogenesis, the PGCs align along the lateral plate mesoderm of the trunk and at the border between the head and trunk mesoderm (Weidinger et al., 1999), and at this stage too, the PGCs are found mainly within domains of *sdf*-1a expression (Figure 1F). At later stages, when the PGCs cluster at the bilateral positions, strong expression of the ligand is observed in the lateral plate mesoderm where the PGCs are located (Figures 1H and 1N). During the last step of migration, the PGCs migrate posteriorly to the position where the gonad develops. Consistently, a high level of *sdf*-1a RNA is detected at this position as well (Figure 1J). In conclusion, we find a strong correlation between the position of the PGCs at different stages of their migration and the tissues expressing high levels of *sdf*-1a transcripts.

If *sdf*-1a indeed acts as an attractant for the PGCs, then one would expect that the dynamics of its expression pattern would mirror the migration pattern of these cells. To test this notion, we looked more closely at the expression pattern of the ligand at several stages relative to concurring steps of PGC migration.

During early somitogenesis stages, the PGCs which align along the tissues that express *sdf*-1a in the trunk (steps IIIa and IIIb in Weidinger et al., 1999; Figure 1F) migrate toward the clustering positions (steps IV and V in Weidinger et al., 1999; Figure 1F, arrows). Consistent with a role for SDF-1a in directing this migration, we find that its RNA is strongly expressed in a large number of cells at the clustering position (Figure 1K). In addition, in parallel to PGC migration toward the lateral clustering sites (Figure 1F, horizontal arrows pointing left and right) a reduction in the expression of the ligand at the first somite is observed while the expression in lateral positions at the same anteroposterior level is enhanced (compare Figures 1F and 1K).

This presumably leads to cell migration toward the adjacent lateral positions expressing high levels of *sdf*-1a.

We then investigated another step of PGC migration where the PGCs migrate posteriorly away from the position where they initially cluster; that is, from the level of the first three somites toward the anteroposterior level of the eighth to tenth somites (step VI in Weidinger et al., 1999, 2002). At this stage too, the expression of the ligand

Fig. 1: For figure legend, see opposite page.

Fig. 1: Expression Patterns of cxcr4b and sdf-1a
(A, C, E, and G) The distribution of the CXCR4b transcripts in wild-type embryos from gastrulation to mid-somitogenesis stages. (A and C) During early gastrulation the mRNA of the receptor is uniformly distributed. (E and G) Tissue-specific pattern is evident during somitogenesis stages when the PGCs coexpress cxcr4b and a PGC-specific RNA, nanos-1. (I) The expression of the receptor persists in the PGCs after they arrive at the region of the gonad (arrowhead).
(B, D, F, and H) The expression pattern of sdf-1a mRNA relative to the position of the PGCs. (B and D) Expression of sdf-1 mRNA during early gastrulation. (F) sdf-1a expression during early somitogenesis. Depending on their position at this stage, the PGCs migrate laterally or anteriorly (arrows). (H) During somitogenesis, sdf-1a is expressed along the border of the trunk mesoderm, in the somites, and in specific domains in the head. (J) By the end of the first day of development, sdf-1a is expressed in the somites, in the brain, and along the pronephric duct. In the region where the gonad develops, a larger number of cells express sdf-1a (arrowhead).
(K–M) Alterations in sdf-1a expression during somitogenesis.
(N) sdf-1a is expressed in the lateral plate mesoderm of the trunk lateral to the domain expressing pax8.
(O and P) Two examples showing that the position of the PGC clusters overlaps with the anterior border of high sdf-1a expression along the trunk mesoderm.

is in perfect agreement with its proposed role in directing the PGCs' route of migration. Here, *sdf*-1a expression in the lateral plate mesoderm progressively recedes from the position of the first somites toward more posterior somites in dynamics identical to that observed for the posteriorly migrating PGCs (Figures 1K–1M; Weidinger et al., 1999). This striking correlation between the expression pattern of the ligand and the position of the PGCs can be visualized in double stainings where the anterior border of *sdf*-1a coincides with the position of the migrating cell cluster (Figure 1O). This point is clearly demonstrated in Figure 1P where residual *sdf*-1a expression can still be detected anterior to the PGC cluster that has migrated toward the region expressing a higher level of the ligand.

Alterations in the Expression Pattern of *sdf*-1a in Mutant Embryos Are Paralleled by Abnormal PGC Migration

Based on the analysis of PGC migration in mutant embryos that exhibit specific differentiation defects, we have previously demonstrated that normal development of certain structures within the embryo is essential for proper PGC migration (Weidinger et al. 1999 and Weidinger et al. 2002). Therefore, we set out to reexamine some of those mutants in an attempt to correlate the PGC migration defects with possible altered expression pattern of the *sdf*-1a gene in these embryos.

The *spadetail* (*spt*) gene, encoding a T box protein, is important for normal development of trunk paraxial mesoderm (Griffin et al., 1998). In *spt* mutant embryos, the PGCs do not align along the border between the head and the trunk mesoderm (step IIIa in Weidinger et al., 1999), and as a result, some PGCs arrive at anterior positions where they eventually cluster (Figure 2D; Weidinger et al., 1999). Indeed, the abnormal PGC migration pattern observed in *spt* mutant embryos coincides with the altered expression pattern of *sdf*-1a. Specifically, at the stage when the PGCs normally align

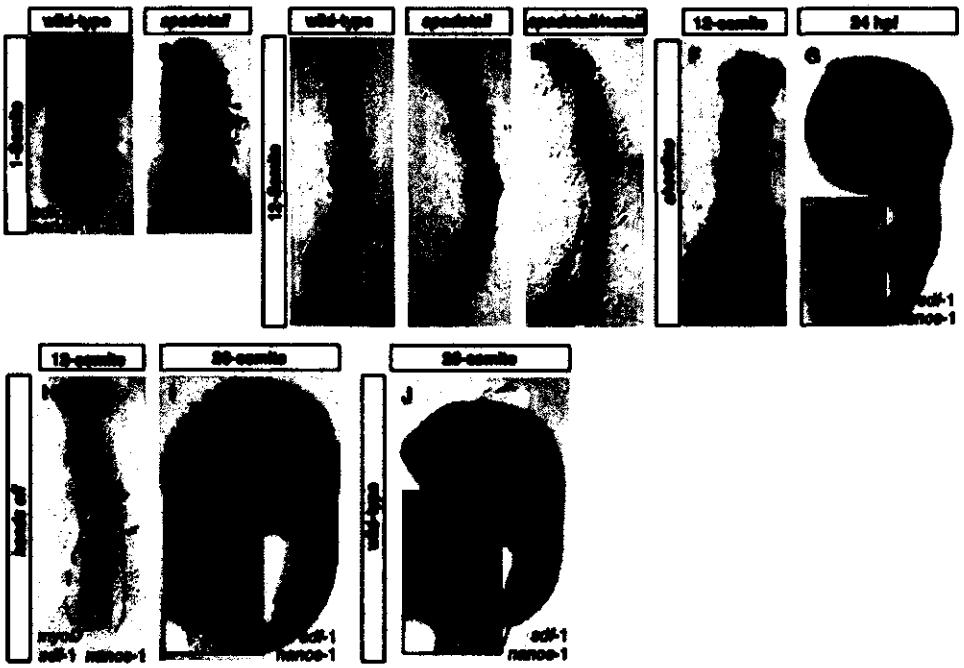

Fig. 2: A Correlation between Specific Alterations in the Expression Pattern of sdf-1a in Mutant Embryos and the Direction of PGC Migration (A) In wild-type embryos at 1 somite stage, the PGCs are found along the borders of the trunk mesoderm where sdf-1a is expressed. (B) In spadetail embryos, the expression of sdf-1a along the border between the head and the trunk mesoderm is eliminated, thereby allowing some cells to reach the head (arrowheads). (C) In wild-type embryos at 12 somite stage, most of the PGCs are found in two cell clusters around the level of the fourth somite. (D) At the same stage in spadetail embryos, PGCs that arrived at the head cluster ectopically in a region that exhibits a high level of sdf-1a expression. (E) In spadetail/notail double mutants, sdf-1a expression in the lateral plate mesoderm of the trunk is severely reduced, and consequently no clustering occurs at the normal position. (F) Posterior expression of sdf-1a in chordino mutant embryos (arrowheads point at posterior PGCs). (G) In 24-hour-old chordino embryos, the ectopic cells are concentrated in regions expressing high levels of the ligand. (H) In hands off mutant embryos, the expression of sdf-1a at the trunk lateral plate mesoderm is reduced (arrow). (I) During subsequent somitogenesis stages in hands off mutants, sdf-1a expression continues to decline and a dispersed anterior PGC cluster is observed. (J) sdf-1a in a 24-hour-old wild-type embryo relative to the PGCs.

between the head and the trunk mesoderm, *sdf*-1a is not expressed along this border as it is in wild-type embryos, thus allowing some cells to reach anterior locations in the embryo (arrowheads in Figure 2B). Consistent with the proposed role for SDF-1a as an attractant for the PGCs, while clustering occurs at the normal positions, the ectopic cells that arrive at the very anterior positions are concentrated in a region where high levels of *sdf*-1a mRNA can be detected (arrowhead in Figure 2D).

As we previously described, in *spadetail* and *notail* (*ntl*) (*spt;ntl*) double mutant embryos, differentiation of the somatic tissue at the clustering position is defective as determined by marker gene expression, and consequently, no PGC clustering occurs at the normal position (Weidinger et al., 2002). In agreement with the proposed role for SDF-1a in attracting PGCs toward the clustering point, very low levels of *sdf*-1a RNA

are detected at this position in *spt;ntl* mutant embryos (compare Figure 2E and Figure 2C).

Loss of function of the zebrafish chordin homolog *chordino*, a BMP antagonist, leads to an expansion of ventral-posterior tissues (Hammerschmidt et al. 1996 and Schulte-Merker et al. 1997). In *chordino* (*din*) mutant embryos, starting at early somitogenesis stages, more PGCs are found in the posterior positions (Weidinger et al., 1999). Unlike the situation in wild-type embryos, these cells never leave this region and are found in the tail of 24-hour-old mutant embryos. The distribution of *sdf*-1a provides the basis for the *din* PGC phenotype. Here, instead of the posterior gap in *sdf*-1a expression observed in wild-type embryos (Figures 2A and 2C), *sdf*-1a is strongly expressed in the posterior-most part of the mutant embryos (Figure 2F). This result is therefore consistent with the idea that in wild-type embryos PGCs migrate toward more anterior positions where the ligand is expressed and eventually arrive at the clustering position. The strong expression of the ligand in posterior tissues of *din* mutant embryos therefore prevents the anterior migration of PGCs and consequently they remain in the tail (Figures 2F and 2G). While we do not know the basis for the ectopic posterior *sdf*-1a expression, a contributing factor to this phenomena could be the abnormal cell movements in this mutant (Myers et al., 2002). Specifically, the increase in nonconverging ventral cells in this mutant could interfere with the elimination of the ventral *sdf*-1a expression.

The last step in PGC migration that we have analyzed in mutant embryos is the migration of the cluster from the original clustering site at the level of the first somites toward a more posterior position as well as anterior migration of trailing posterior cells toward the region where the gonad develops. We have previously found that in *hands off* mutant embryos, which carry a mutation in the bHLH transcription factor *hand* 2, the migration of the PGC cluster posteriorly is disrupted (Weidinger et al. 2002 and Yelon et al. 2000). We therefore examined the expression of *sdf*-1a in this mutant. From the 12 somite stage and onward, the expression domain of *sdf*-1a becomes increasingly restricted in *hands off* mutant embryos (compare Figure 2H, arrow, with Figure 2C), but this has yet no effect on the cells that cluster normally and align along the line defined by the expression pattern of the gene. It is only at later stages that the level of *sdf*-1a is reduced to a degree that leads to abnormal migration (compare Figure 2I with Figure 2J). The severe decline in the level of *sdf*-1a mRNA in the region where the gonad should develop presumably results in inability of the cluster to migrate posteriorly and of trailing posterior cells to migrate anteriorly.

In conclusion, the expression analysis described above in wild-type and mutant embryos shows a remarkable correlation between the positions at which *sdf*-1a is expressed at higher levels and positions toward which the PGCs migrate during the first 24 hr of zebrafish embryonic development. To examine more directly whether CXCR4b and SDF-1a are functionally essential for normal PGC migration, we studied the effect of knocking down the activity of each one of them on the migration process.

Knockdown of the Activity of CXCR4b and of the SDF-1a Ligand Result in Severe PGC Migration Defects

To determine how a reduction in the amount of CXCR4b and the chemokine SDF-1a would affect PGC migration, we injected embryos with morpholino antisense oligonucleotides (Nasevicius and Ekker, 2000) directed against either one of these genes and examined the resulting PGC distribution by in situ hybridization using different PGC markers. Injection of these morpholino oligonucleotides (two different oligonucleotides were used for each gene with similar results) resulted in a dramatic migration phenotype where at mid somitogensis stages most of the PGCs were randomly scattered in the embryo (Figures 3A–3C).

Analysis of the morpholino-injected embryos using different markers demonstrates that the abnormal phenotype reflects primarily migration rather than a PGC or somatic differentiation defect. First, the ectopic PGCs show normal expression of a PGC RNA marker, the zebrafish *h1m*RNA (Figures 3A–3C; Müller et al., 2002). The regulation of *h1m*RNA tissue-specific expression is unique considering that it is not incorporated into the zebrafish germ plasm and is not subjected to "degradation-protection" regulation as seen in other PGC-specific genes (Köprunner et al. 2001; Müller et al. 2002 and Wolke et al. 2002). At the same time, the PGCs exhibit normal expression of RNA molecules like *nanos*-1 (Figures 3J–3L) and *vasa* (data not shown) that characteristically reside in the germ plasm. Further, when a fusion RNA between GFP and the 3′ untranslated region (3′-UTR) of the *nanos*-1 gene was coinjected with the antisense oligonucleotides, the abnormally migrating cells were capable of specifically protecting this RNA from degradation, thereby obeying to the degradation-protection rule of regulation as do the PGCs in control embryos (Figures 3D–3F). Last, the knocked down PGCs contained perinuclear granules, a characteristic feature of germ cells (Eddy 1974; Eddy and Ito 1971; Hay et al. 1988; Knaut et al. 2000; Schisa et al. 2001 and Strome and Wood 1982). This point was demonstrated by expressing a Vasa-GFP fusion protein in the PGCs and observing proper subcellular localization to the perinuclear granules whether the cells arrived at the gonad region (Figure 3G; Wolke et al., 2002) or were found at ectopic positions (Figures 3H and 3I).

While the reduction in the activity of SDF-1a or CXCR4b results in severe migration defects, it is formally possible that this effect is indirect, resulting from defects in the differentiation of somatic tissues that normally support PGC migration. Several lines of evidence argue against this possibility. First, despite the adverse effect on PGC migration, the morphology and development of the embryos (e.g., Figures 3A–3I) appeared similar to embryos injected with the control morpholino oligonucleotides. To substantiate this point more rigorously, we analyzed the injected embryos using different molecular markers expressed in somatic tissues. Under the experimental conditions that affected PGC migration, we could not detect any obvious defects reflected by alteration in gene expression. For example, the lateral border of the mesoderm, the clustering point at the anterior trunk, the developing somites, and specific structures in the head were expressing *sdf*-1a in a pattern similar to that in control embryos (Figures 3J–3L). Using other markers for somatic structures such as *Myo* D (somites and adaxial cells), *Pax* 8, and *Pax* 2.1 (midhindbrain boundary and

pronephros), we did not detect any obvious abnormalities in the injected embryos (data not shown).

To prove that the activity of CXCR4b is indeed required specifically in the PGCs, we coinjected embryos with a morpholino against the receptor and different

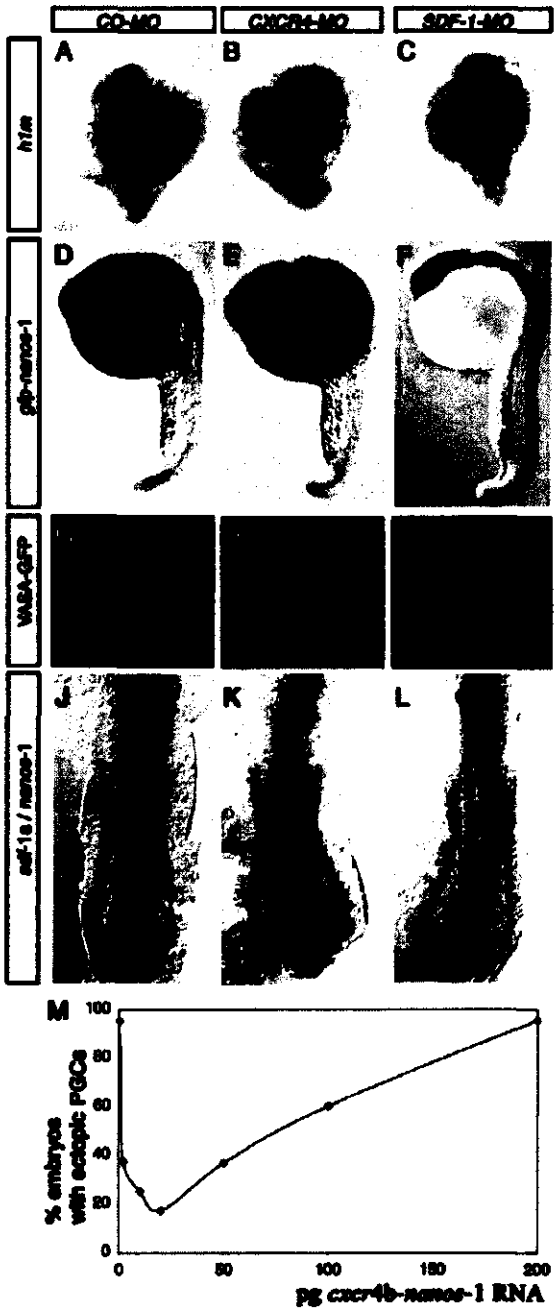

Fig. 3: Knockdown of CXCR4b and SDF-1a by Morpholinos Disrupts PGC Migration without Affecting Their Identity
(A–C) h1m RNA is normally expressed in the PGC clusters of 14 somite stage control embryos as well as in ectopic PGCs in embryos injected with a morpholino directed against cxcr4b or sdf-1a.
(D–F) PGCs in embryos injected with the control morpholino and gfp-nanos-1 mRNA specifically protect the injected mRNA, similar to ectopic PGCs in embryos injected with morpholino directed against cxcr4b or sdf-1a.
(G–I) A GFP-Vasa fusion protein is subcellularly localized to perinuclear granules in the PGCs of control embryos similar to the localization in ectopic cells in embryos injected with morpholino directed against cxcr4b, or sdf-1a.
(J–L) Normal somatic development in embryos injected with the control morpholino or morpholinos against cxcr4b or sdf-1a as revealed by the expression of sdf-1a mRNA.
(M) The PGC migration phenotype in embryos injected with morpholinos against the CXCR4b is reversed by specific expression of the receptor in the PGCs.

amounts of *cxcr* 4b-*nanos*-1 RNA lacking the morpholino binding site. This way we targeted specific expression of CXCR4b to the PGCs in embryos globally depleted of the protein (see Experimental Procedures and Köprunner et al., 2001). Indeed, small amounts of this RNA were sufficient to reverse the morpholino-induced phenotype, allowing all of the PGCs to arrive at the region of the gonad in most of the embryos (Figure 3M). Interestingly, when high amounts of the RNA were injected, PGC migration appeared abnormal, presumably due to basal level of signaling of the overexpressed receptor.

Taken together, these experiments show that CXCR4b activity in PGCs is important for their directional migration. This finding allows us now to follow the chemokine-dependent migration in live embryos and to evaluate the effects of loss of this signaling pathway on cell behavior.

Alterations in the Level of the SDF-1 Signaling Interferes with Directional PGC Migration

To account for the abnormal distribution of the cells in which the chemokine signaling level was altered, we first analyzed PGC migration in knocked down embryos at low magnification using time-lapse microscopy. A detailed description of the effects of this treatment on the dynamics of cell behavior and cytoskeletal architecture will be presented elsewhere (M.R-F., M.D., and E.R., unpublished), but a clear phenotype is evident already at this level of resolution. The PGCs in which the chemokine signaling was knocked down were capable of migrating relative to their neighboring somatic cells (Figures 4A–4C). However, in sharp contrast to the directed migration of wild-type PGCs toward the borders of the trunk mesoderm, the knocked down cells exhibit irregular nondirectional movement (Figures 4A–4C and see Supplemental Movies S1–S3 at http://www.cell.com/cgi/content/full/111/5/647/DC1). As a result, the cells remained in regions that they normally vacate, such as the segmental plate and the notochord, and exhibit no specific anteroposterior positioning.

To observe the cellular morphology and movement at a higher resolution, individual cells whose membrane was labeled with GFP were examined at higher magnification. Here again, a clear difference was observed between the knocked down PGCs and the control. Although PGCs depleted of CXCR4b generated lamellipodia, in striking contrast to control cells, these cellular protrusions extended in multiple directions, leading to a nonpolarized appearance of the cells (Figures 5A and 5B and see Supplemental Movies S5 and S6 at http://www.cell.com/cgi/content/full/111/5/647/DC1). Similar apolar cellular behavior was observed when the SDF-1a level was reduced (data not shown).

Fig. 4: Alterations in the Level of the SDF-1a Signaling in the PGCs Result in Nondirected Cell Migration as Revealed by Time Lapse Analysis
(A) In embryos injected with control morpholino, the PGCs migrate toward the border between the head and the trunk mesoderm.
(B and C) Inhibiting the translation of the cxcr4b or sdf-1a mRNA results in random migration with cells arriving at ectopic positions.
(D) Overexpression of SDF-1a in the PGCs leads to a similar phenotype. In each image, four time points are shown, representing 3 hr of development starting at late gastrulation (80% epiboly).
In each image, the filmed embryo is shown at the end of the first day of development.
In the experimental embryos, two individual cells were artificially colored in red and green (B–D).
The filmed embryos were injected with gfp-nanos-1 mRNA, leading to GFP expression in their PGCs, and express GFP under the control of the goosecoid promoter, which labels their axial mesoderm (see corresponding Supplemental Movies S1–S4 at http://www.cell.com/cgi/content/full/111/5/647/DC1).

The Chemokine SDF-1 Directs PGC Migration

The migration phenotype described above is consistent with the notion that SDF-1a functions by introducing a bias in the direction of cell migration, leading the PGCs toward the source of the ligand. A prediction of this proposal is that expression of the ligand at high levels within the PGCs themselves would interfere with their ability to detect the endogenous signal. Indeed, overexpression of the ligand in the PGCs results in pronounced migration defects in otherwise wild-type embryos (Figure 4D and see Supplemental Movie S4 at http://www.cell.com/cgi/content/full/111/5/647/DC1). Specifically, PGCs overexpressing the ligand exhibit limited migration relative to their somatic neighbors and form cellular protrusions all around the circumference of the cell. As a result, the PGCs overexpressing SDF-1a are randomly distributed within the embryo (Figure 4D). Interestingly, the morphology of the cellular extensions in this case was different from that of the knocked down cells. PGCs overexpressing the SDF-1a develop longer and thinner cellular extensions (Figure 5C and Supplemental Movie S7), suggesting that high level of CXCR4b activation promotes the stabilization of these protrusions.

In view of these results, coupled with the strong correlation between the expression pattern of SDF-1a and the direction of PGC migration, it is reasonable to consider SDF-1a as the molecule that attracts the PGCs toward their intermediate and final targets. To examine this assumption, we designed experiments in which the endogenous SDF-1a activity was inhibited and tested the ability of exogenously introduced SDF-1a to attract the PGCs to ectopic positions (Figure 6).

To overcome the activity of the endogenous SDF-1a, we inhibited its translation by injecting specific anti-sdf-1a morpholinos into 1 cell stage embryos as well as mRNA encoding EYFP fused to the nanos-1 3′-UTR for the purpose of labeling the PGCs. We then raised the embryos to the 32 cell stage and injected cells with morpholino-resistant sdf-1a mRNA and with mRNA encoding the ECFP protein, thereby generating an ECFP-and SDF-1a-expressing clone of cells. The ability of the EYFP-labeled PGCs to arrive at areas in the embryo expressing high level of SDF-1a (marked by ECFP expression) was monitored and compared to that in control experiments. Indeed, while the cells exhibited random distribution with respect to the ECFP expression in the control experiment in which SDF-1a was knocked down (Figures 6B and 6E), they specifically arrived at regions in which SDF-1a was expressed (Figures 6C and 6E). Furthermore, the ability of the PGCs to respond to the exogenously supplied SDF-1a depended on the expression of CXCR4b. Injection of anti-cxcr 4b antisense oligonucleotides at the 1 cell stage interfered with the directed PGC migration toward the SDF-1a source (Figures 6D and 6E).

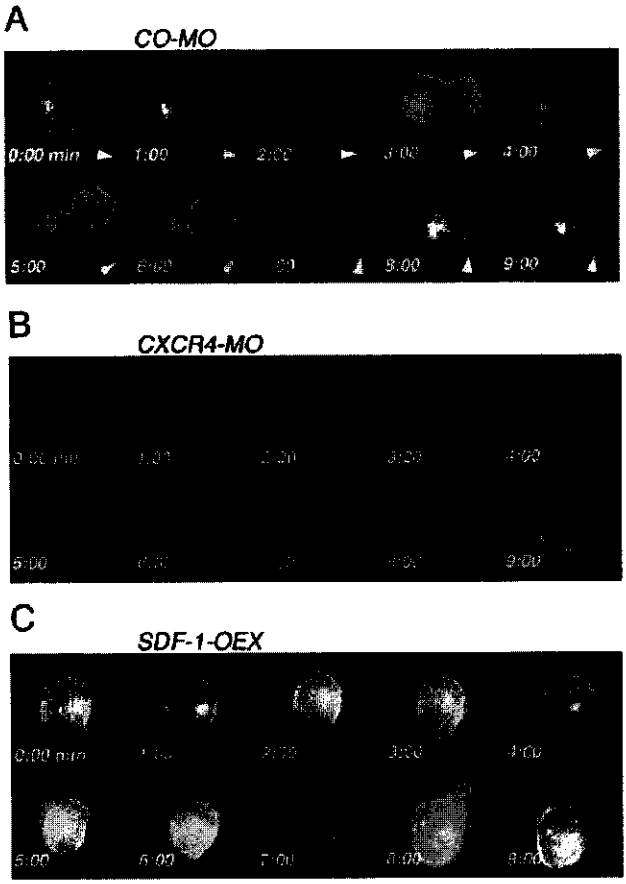

Fig. 5: Alterations in the Level of the SDF-1a Signaling Affects PGC Polarity and Cellular Morphology as Revealed by High-Magnification Time Lapse Analysis
(A) Migrating PGCs are characteristically polarized, exhibiting protrusions that are extended primarily in the direction of their movement (indicated by arrowheads) and are normally elongated in shape.
(B) Inhibition of cxcr4b translation leads to apolar cell morphology.
(C) Overexpression of SDF-1a in the PGCs similarly leads to loss of polarity with cellular extensions that appear longer and more elaborate.
In each image, snapshots from a time lapse video recorded over 9 min (see Supplemental Movies S5--S7 at http://www.cell.com/cgi/content/full/111/5/647.DC1).

DISCUSSION

Starting their migration from random positions within the embryo, zebrafish PGCs execute a number of migration steps, passing through several intermediate targets on their way toward two clustering positions on either side of the body axis in the region where the gonad will be formed. In this study, we provide strong evidence for the involvement of the chemokine SDF-1a in providing directional information to the migrating cells.

The expression pattern of SDF-1a perfectly overlaps with the positions toward which the PGCs migrate, and alterations in the expression pattern of SDF-1a in mutant embryos lead to a predictable migration phenotype. Furthermore, reduction in the level of SDF-1a or of its putative receptor CXCR4b results in migration defects. Last, we show that migration of the PGCs can be redirected toward sites of ectopically expressed SDF-1a. Our interpretation of these findings is that SDF-1a acts as a natural chemoattractant for zebrafish PGCs. A formal possibility is that SDF-1a is not the

actual attractant, but rather that the chemokine signal leads to the production of another molecule that acts as the actual cue for the PGCs. We consider the idea of such a relay mechanism unlikely. First, consistent with our model, in a wide range of experimental systems, SDF-1 has been shown to act as the chemotactic factor per se, directing cells toward their target. Second, we observe exceedingly tight temporal relationships between the RNA expression of SDF-1a and the cellular response, a finding that renders a relay mechanism less likely. Third, our ability to redirect cells toward ectopic positions by applying SDF-1a to these positions and to misguide the PGCs by expressing the ligand within them is less compatible with a relay mechanism in which a vast array of cell types would be required to be capable of mediating this process. Last, restoring the activity of CXCR4b specifically in the PGCs was sufficient to reverse the abnormal migration phenotype in embryos globally depleted for the receptor, providing further support to the notion that the activity of the receptor is required in the migrating cells themselves.

The PGCs appear to be very sensitive to changes in the level of SDF-1a protein, which is reflected by the tight correlation between high *sdf*-1a expression and the position of the cells or their direction of migration. This high sensitivity of the cells to small changes in the attractant level is consistent with studies revealing particularly high potency of SDF-1-mediated lymphocyte chemoattraction relative to other chemokines (Bleul et al., 1996a). Such high sensitivity of the responding cells to SDF-1a allows precise migration of PGCs toward their target despite complex and sometimes opposing morphogenetic movements that occur at the same time during development (Weidinger et al., 1999). The robust manner in which SDF-1a guides PGCs was demonstrated here by its ability to attract PGCs to ectopic locations. Conversely, PGCs transplanted into the animal pole of the zebrafish blastula, a region normally devoid of PGCs, are able to arrive at the correct position by the end of the first day of development (Ciruna et al., 2002), notwithstanding the ectopic origin of migration and the coinciding complicated gastrulation movements in the embryo. Analogously, the potency of SDF-1 enables efficient stem cell homing and mobilization (Peled et al. 1999b and Petit et al. 2002) and leuckocyte traffic (Aiuti et al. 1997; Baggiolini 1998; Bleul et al. 1996a and Zou et al. 1998). It will be interesting to determine if the analogy between zebrafish PGC migration and leukocyte trafficking can be extended to other levels such as regulated cell adhesion, which plays an important role in targeting of leukocytes (Campbell et al. 1998; Mazo et al. 1998; Peled et al. 1999a and Springer 1994).

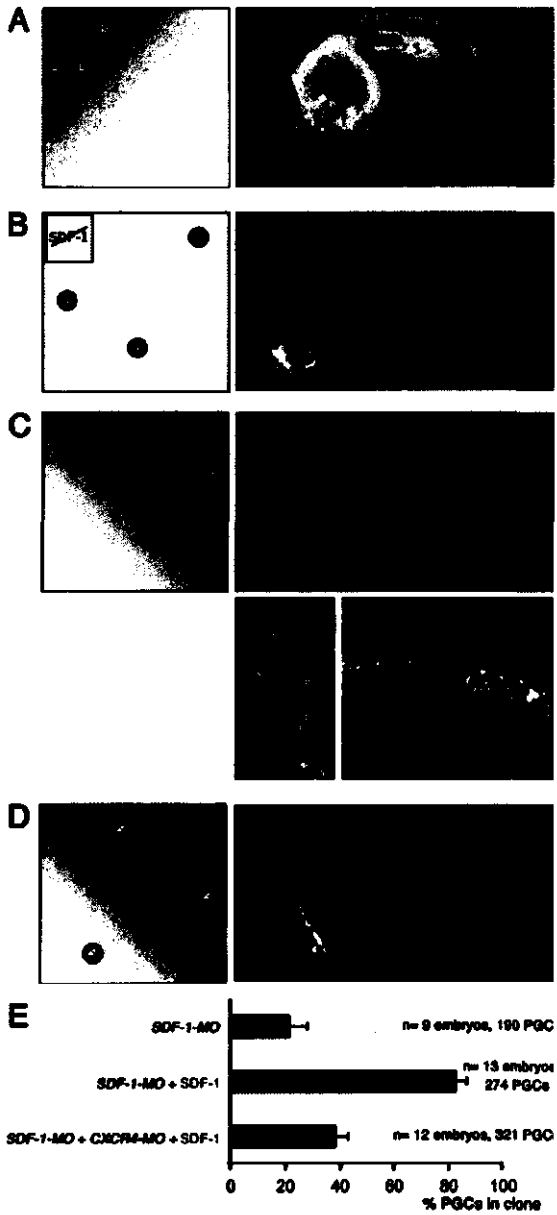

Fig. 6: PGCs Are Attracted toward an Ectopic Source of SDF-1a (A) An embryo injected with control morpholino and eyfp-nanos-1 RNA at 1 cell stage and ecfp-globin at 32 cell stage. The EYFP-labeled PGCs (black) are found at the normal position. (B) An embryo injected at the 1 cell stage with SDF-1a-MO and eyfp-nanos-1 mRNA, followed by a later injection of ECFP mRNA into cells at the 32 cell stage. The PGCs are randomly distributed with respect to the ECFP-expressing clone of cells (pseudocolors of red and white represent high and higher levels of ECFP expression, respectively). (C) Top: an embryo injected at the 1 cell stage with SDF-1a-MO and eyfp-nanos-1 mRNA followed by injection of cells at the 32 cell stage with ECFP and morpholino-resistant sdf-1a mRNA. The PGCs are preferentially found in close proximity to the ECFP- and SDF-1a-expressing cells (pseudocolors represent the levels of ECFP- and therefore SDF-1a expression). Bottom: high-magnification pictures of the head region (left) and the notochord (right) of a different embryo treated as above. (D) An embryo injected at the 1 cell stage with SDF-1a-MO, CXCR4b-MO, and eyfp-nanos-1 mRNA followed by injection of cells at the 32 cell stage with ECFP and morpholino-resistant sdf-1a mRNA. In these embryos, many PGCs are randomly distributed relative to the ECFP-expressing clone of cells. (E) Quantitative analysis of the experimental results. In embryos treated as described in (B), approximately 20% of the somatic cells exhibit ECFP expression, and a similar proportion (yellow bar, 21.5% ± 6.4%) of PGCs were found within the clone. In embryos treated as described in (C), the PGCs are preferentially found within the ECFP/SDF-1a-expressing clone (red bar, 82.5% ± 3.9%, $p < 0.001$ in t test). The attraction of PGCs toward the SDF-1a clone is inhibited when CXCR4b is knocked down (green bar, 38.4% ± 4.5%, $p < 0.001$ in t test).

Unlike other chemokines whose function and expression are centered around their role in leukocyte trafficking, both SDF-1 and its receptor CXCR4 were found to be expressed in a wide variety of cell types and tissues. Consistently, mice lacking either the SDF-1 or its receptor exhibit defects that extend beyond the immune system, revealing roles for this chemokine in the genesis of the circulatory and central nervous systems (Nagasawa et al. 1996; Tachibana et al. 1998 and Zou et al. 1998). Our finding

that PGC migration depends on directional signaling by SDF-1a allows us to model these processes in a more accessible system at a higher resolution. Furthermore, although embryos in which SDF-1a or CXCR4b activity was knocked down did not exhibit increased lethality or obvious somatic defects, given the widespread expression of these genes, a thorough analysis of behavior of other cell types may reveal additional requirements for SDF-1a and its receptor in zebrafish.

Our initial observations indicate that the activity of the chemokine signaling pathway is not required for the formation of the cellular extensions characteristic of migrating cells, nor is it essential for the ability of the cell to move. Rather, the most prominent feature of cells in which SDF-1a function is inhibited is the loss of stable cell polarity and directed cell migration. Consistent with our findings, G protein-linked signaling has been shown to polarize cells such as neutrophils that respond to chemoattractants by polarizing the distribution of PIP_3 and directed migration (Servant et al., 2000). The most detailed understanding of establishment of cell polarity in chemotaxis was obtained in *Dictyostelium discoiddeum* (reviewed in Iijima et al. 2002 and Parent and Devreotes 1999). In this model system, directional migration toward the chemoattractant is also controlled by asymmetric activation of a G protein-coupled receptor. This is translated into asymmetrical distribution of PIP_3 by spatially and temporally regulated production and subsequent degradation of this phosphoinositide (Funamoto et al. 2002 and Iijima and Devreotes 2002). In accordance with our findings, an important conclusion derived from studying chemotaxis in *Dictyostelium* is that while directional sensing depends on receptor activation, the actual movement of the cell is independent of it (Parent and Devreotes, 1999). An interesting avenue of research would be to determine whether these striking similarities between *Dictyostelium* chemoattraction and PGC migration includes the molecular cascade downstream of the receptor. These questions can be addressed now *in vivo* in the context of a live vertebrate at a resolution approaching that obtained in *Dictyostelium*.

As mentioned above, the process of PGC migration takes place in many different organisms. The question of whether PGC migration in these organisms is regulated by molecular mechanisms similar to those described here for the zebrafish remains open. While strong tools are available for following PGC migration in mouse (Anderson et al. 2000 and Molyneaux et al. 2001), they have not yet been applied to study mice deficient for SDF-1 or for CXCR4. In addition, it is not clear whether molecules that are functionally homologous to SDF and its receptor are present in *Drosophila*. The question of conservation is especially intriguing in the chick (reviewed in Niewkoop and Sutasurya, 1979). In this organism, following their segregation, the PGCs penetrate the vascular system and are found in the extraembryonic blood vessels and then in the blood circulation of the embryo proper. The PGCs then leave the blood vessels and begin to penetrate the gonadal epithelium. This route of PGC migration is noticeably reminiscent of leukocyte trafficking, where the cells migrate into and out of blood vessels to arrive at their target. The involvement of attracting signals directing PGC migration in the chick has been suggested, for example, by showing that an ectopically transplanted target tissue is capable of attracting and then being colonized by PGCs (Kuwana and Rogulska, 1999). In addition to seeking a generalized concept underlying the findings presented here and PGC migration in other organisms, it would be necessary to determine the relationships

between SDF-1 signaling and the corresponding pathways that are involved in PGC migration in other organisms (Starz-Gaiano and Lehmann 2001; Wylie 1999 and Wylie 2000). For example, the possible relevance of lipid-metabolizing enzymes whose function is important for PGC migration in *Drosophila* (Starz-Gaiano et al. 2001; van Doren et al. 1998 and Zhang et al. 1996) should be investigated in the fish. Similarly, the involvement of specific cell-cell and cell-matrix adhesion molecules shown to participate in mouse PGC migration (Anderson et al. 1999 and Bendel-Stenzel et al. 2000) should also be examined in the zebrafish system.

Besides their importance in normal processes such as leukocyte migration and organ development, SDF-1 and CXCR4 have been shown to be involved in several pathological conditions. CXCR4 plays a central role in T cell accumulation in rheumatoid arthritis synovium (e.g., Buckley et al. 2000; Gu et al. 2002 and Nanki et al. 2000), it determines the metastatic destination of tumor cells (Muller et al., 2001), and it acts as a coreceptor for the HIV-1 entry and membrane fusion with the target cell (Muller et al., 2001). Using the available genetic and genomic tools in zebrafish as a model for these diseases would enhance our understanding of these disorders. Moreover, compounds that may provide a possible prospective treatment for conditions of this kind (e.g., Matthys et al. 2001; Schols et al. 1997a and Schols et al. 1997b) could be screened for, using PGC migration as an *in vivo* assay for the SDF-1-CXCR4 signaling pathway (Peterson et al., 2000).

EXPERIMENTAL PROCEDURES

Cloning of CXCR4b and SDF-1a

The *cxcr* 4b (Chong et al., 2001) cDNA was PCR amplified from early somitogenesis AB cDNA using the primers 5′-CCGGACTTATTGCGCCTTT-3′ and 5′-TGATTTTTAGGTTTTATTATGAAATGG-3′. Another *cxcr* 4 gene, the *cxcr* 4a (Chong et al., 2001), has been previously described. Based on the expression pattern of this gene and the fact that inhibition of its translation does not affect PGC migration, we consider it unlikely that it plays a role in this process. The cDNA of *sdf*-1a (GenBank accession number AY147915) was amplified from early somitogenesis AB cDNA using the primers 5′-CAGTGCGGATCTCTTCTTCA-3′ (forward) and 5′-AAACACGGAGCAAACAGGAC-3′ (reverse) based on expressed sequence tags (ESTs) sequences identified using BLAST search for molecules homologous to the mouse *sdf*-1. An EST for another *sdf*-1-related gene was identified (termed *sdf*-1b, accession number BM070896). Based on the expression pattern of this gene and the fact that its overexpression in the PGCs did not affect their migration, we consider it unlikely that this gene is involved in PGC migration.

Knockdown Experiments

Two non-overlapping morpholino oligonucleotides were used to inhibit the translation of *cxcr*4b with similar results: *R4b*-1-*MO* TGCTCAAAAAGGTGCAATAAGTCCG and *R4b*-2-*MO* AAATGATGCTATCGTAAAATTCCAT. As a control, a morpholino oligonucleotide that includes four mismatching bases was injected: *R4b*-2-*CO*-*MO* AATTGA AGCTATCGTAATATTGCAT. To inhibit the translation of *sdf*-1a mRNA, two different morpholino oligonucleotides were used with similar results: *SDF-1a*-1-*MO* 5'-CTACTACGATCACTTTGAGATCCAT-3' and *SDF-1a-2-MO* 5'-TTGAGATCCATGTTTGCAGTGTGAA-3'.

For the knockdown experiments, 0.4 pmol of these morpholino oligonucleotides dissolved in 10 mM HEPES (pH 7.6) were injected into 1 cell stage embryos.

Construction of RNA Expression Vectors and RNA Synthesis

gfp-nanos-1 is a construct that includes the mmGFP open reading frame (ORF) (Siemering et al., 1996) fused to the 3'-UTR of the *nanos*-1 gene as previously described (Korpuner, 2001). Injection of RNA generated from this construct results in specific GFP expression in the PGCs.

eyfp-nanos-1-3'-UTR is a construct similar to *gfp-nanos*-1, with EYFP replacing the mmGFP ORF.

vasa-gfp-nanos-1 is a construct that encodes a fusion between Vasa and mmGFP ORFs as previously described (Wolke et al., 2002), with the 3'-UTR of *vasa* exchanged for that of the *nanos*-1 gene. The resulting fusion protein is subcellularly localized to perinuclear granules in the PGCs (Wolke et al., 2002).

Farnesylated *egfp-nanos*-1 is a construct that was cloned for the purpose of targeting GFP expression to the membrane of PGCs (Weidinger et al., 2002).

ecfp-globin was made by CEFP cloned into pSP64T. Injection of RNA produced from this construct leads to CEFP expression in cells that inherit it.

For *cxcr* 4b-*nanos*-1, the CXCR4b ORF was fused to the 3'-UTR of *nanos*-1 for the purpose of expressing the protein specifically in PGCs. The coding region of *cxcr* 4b was amplified by PCR using the primers 5'-AAAGGATCCGAACAAAATGGAATTTTACGATAGC-3' and 5'-AAACTCGAGACATGCACACACTAACTCGTCA-3' and was cloned upstream of the *nanos*-1 UTR.

For *sdf*-1a-*nanos*-1, the SDF-1a ORF was fused to the 3'-UTR of *nanos*-1 for the purpose of overexpressing the protein in PGCs. The coding region of *sdf*-1a was

amplified by PCR using the primers 5′-AAAGGATCCAACATGGATCTCAAAGTG-3′ (forward) and 5′-AAACTCGAGTTAGACCTGCTGCTGTTG-3′ (reverse). The *sdf*-1a coding region was cloned into the *gfp-nanos*-1 construct replacing the GFP ORF.

Morpholino-resistant *sdf*-1a-*globin* was used for ectopic expression of SDF-1a in *SDF-1a-MO*-injected embryos. The SDF-1a ORF was amplified using the primers 5′-AAAGGATCCAACATGGACTTGAAGGTCATCGTAGT-3′ (underline, nucleotide exchanges relative to the wild-type *sdf*-1a which should eliminate the binding of the *SDF-1a-MO*-1) and 5′-AAAACTAGTTTAGACCTGCTGCTGTTG-3′. The amplification product that encodes a wild-type SDF-1a was cloned into CEFP-*globin* construct replacing the CEFP ORF.

Phenotypic Rescue and Overexpression Experiments

To express CXCR4b specifically in the PGCs in embryos globally depleted of CXCR4b, embryos were coinjected at 1 cell stage with 0.4 pmol of *R4b-1-MO* and different amounts of *cxcr* 4b-*nanos*-1 and *gfp-nanos*-1 RNA. The injected amounts of *cxcr* 4b-*nanos*-1 were 0, 2, 10, 20, 50, 100, and 200 pg per embryo, and the total amount of injected RNA was supplemented with *gfp-nanos*-1 to 300 pg. For each combination, 40 embryos or more were analyzed. The *cxcr*4b-*nanos*-1 RNA lacks the sequence recognized by the *R4b-1-MO*.

For SDF-1a overexpression in the PGCs, embryos were injected at 1 cell stage with 100 pg *sdf*-1a-*nanos*-1 RNA.

Whole-Mount In Situ Hybridization

One-and two-color 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). The following probes were used: *cmlc* 2(Yelon and Stainier, 1999), *cxcr* 4b (Chong et al., 2001), *myo* D (*myod;* Zebrafish Information Network) (Weinberg et al., 1996), *nos* 1 (Köprunner et al., 2001), *ntl* (Schulte-Merker et al., 1994), *pax* 2.1 (*pax* 2*a*; Zebrafish Information Network) (Krauss et al., 1991), *pax* 8(Pfeffer et al., 1998), and *sdf*-1a (this work).

Time Lapse Analysis of PGC Migration

For the time lapse analysis at low magnification (Figure 4, 10× objective), the PGCs were labeled by microinjection of 150 pg *gfp-nanos*-1 RNA (Köprunner et al., 2001) into embryos obtained by mating wild-type AB females with *goosecoid-GFP*

transgenic males. The embryos were oriented in 1.5% agarose ramps and overlaid with 0.3× Danieau's solution (Westerfield, 1995). The time lapse movies were generated using the Metamorph software (Universal Imaging) controlling a Zeiss Axioplan2 microscope. Pictures were taken at 1 min intervals. For the time lapse analysis at high magnification (Figure 5, 63× objective), wild-type embryos were microinjected with 140 pg of farnesylated-*egfp-nanos-1* RNA leading to GFP expression in the membrane of the PGCs. To obtain faint labeling of somatic cells, 2 pg farnesylated *egfp-globin* was also injected. Pictures were taken at 10 s intervals.

Ectopic Expression of SDF-1a

To express SDF-1a in ectopic positions in the embryo, the embryos were injected at the 1 cell stage with 0.4 pmol *SDF-1a-1 MO,* 0.2 pmol *R4b-2-CO-MO,* and 120 pg YEFP-*nanos*-1-3′-UTR RNA. The embryos were raised to the 32 cell stage and cells were injected with 100 pg morpholino-resistant *sdf*-1a-globin RNA and 100 pg *cefp*-globin RNA. As a control, the embryos were treated at 1 cell stage similarly to the experimental embryos and at 32 cell stage were injected only with 200 pg *cefp*-globin RNA. As a second control, the embryos were injected at 1 cell stage with 0.4 pmol *SDF-1a-MO,* 0.2 pmol *R4b-2-MO,* and 120 pg *eyfp-nanos*-1 and at the 32 cell stage were treated as the experimental embryos. The injected embryos were photographed at around 24 hpf using ECFP and EYFP specific fluorescent filters. Pseudocolors were used to improve the visualization of the fluorescence intensities. The proportion of PGCs in contact with red and white spots that correspond to high and higher intensities, respectively, of ECFP (and therefore of SDF-1a) was counted and the results were analyzed using student's t test.

Additional information regarding experimental procedures is provided in the Supplemental Data at http://www.cell.com/cgi/content/full/111/5/647/DC1.

ACKNOWLEDGEMENTS

This work was supported by grants from the DFG and the Volkswagen-Stiftung to E.R. We thank members of the Raz lab for discussions, Karin Dumstrei and Krasimir Slanchev for comments on the manuscript, and Peter Gruss and Herbert Jäckle for help and encouragement.

Chapter 3

Germ Cell Migration in Zebrafish Is Dependent on HMGCoA Reductase Activity and Prenylation

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This chapter has been published as Thorpe et al. in Dev Cell 6:295-302 (2004)

Germ Cell Migration in Zebrafish Is Dependent on HMGCoA Reductase Activity and Prenylation

ABSTRACT

Hydroxymethylglutaryl coenzyme A reductase (HMGCoAR) is required for isoprenoid and cholesterol biosynthesis. In *Drosophila*, reduced HMGCoAR activity results in germ cell migration defects. We show that pharmacological HMGCoAR inhibition alters zebrafish development and germ cell migration. Embryos treated with atorvastatin (Lipitor) exhibited germ cell migration defects and mild morphologic abnormalities. The effects induced by atorvastatin were completely rescued by prior injection of mevalonate, the product of HMGCoAR activity, or the prenylation precursors farnesol and geranylgeraniol. In contrast, squalene, a cholesterol intermediate further down the pathway, failed to rescue statin-induced defects. Moreover, pharmacologic inhibition of geranylgeranyl transferase 1 (GGT1) protein prenylation activity also resulted in abnormal germ cell migration. Thus, our pharmacological inhibition-and-rescue approach provided detailed information about the elements of isoprenoid biosynthesis that contribute to germ cell migration. Together with data from *Drosophila* (Santos and Lehmann, 2004, this issue), our results highlight a conserved role for protein geranylgeranylation in this context.

INTRODUCTION

A key regulatory step in the synthesis of cholesterol is the reduction of hydroxymethylglutaryl-Coenzyme A to mevalonate by the enzyme Hydroxymethylglutaryl coenzyme A reductase (HMGCoAR) (Figure 1) (Sever et al., 2003). Evidence from studies in *Drosophila* indicates that there are cholesterol-independent developmental defects associated with genes in the mevalonate pathway. In flies lacking HMGCoAR, primordial germ cells (PGCs) fail to reach their correct target in the mesoderm (Van Doren et al., 1998). Importantly, cholesterol or its derivatives do not participate in producing the signal required for germ cell migration, because *Drosophila* lacks a number of enzymes necessary for de novo cholesterol synthesis (Santos and Lehmann, 2004).

PGC migration in zebrafish can be divided into several steps that appear to rely on the integrity of specific somatic structures. During somitogenesis, bilateral clusters of PGCs migrate posteriorly until they reach the anterior of the yolk extension, the position where the gonad develops (Weidinger et al., 2002). Recently, the chemokine SDF-1a (stromal cell derived factor 1a) (Doitsidou et al., 2002) and its receptor CXCR4b (Doitsidou et al. 2002 and Knaut et al. 2003) were identified as molecules essential for proper migration of zebrafish PGCs. When expression of cxcr4b or its ligand is inhibited,

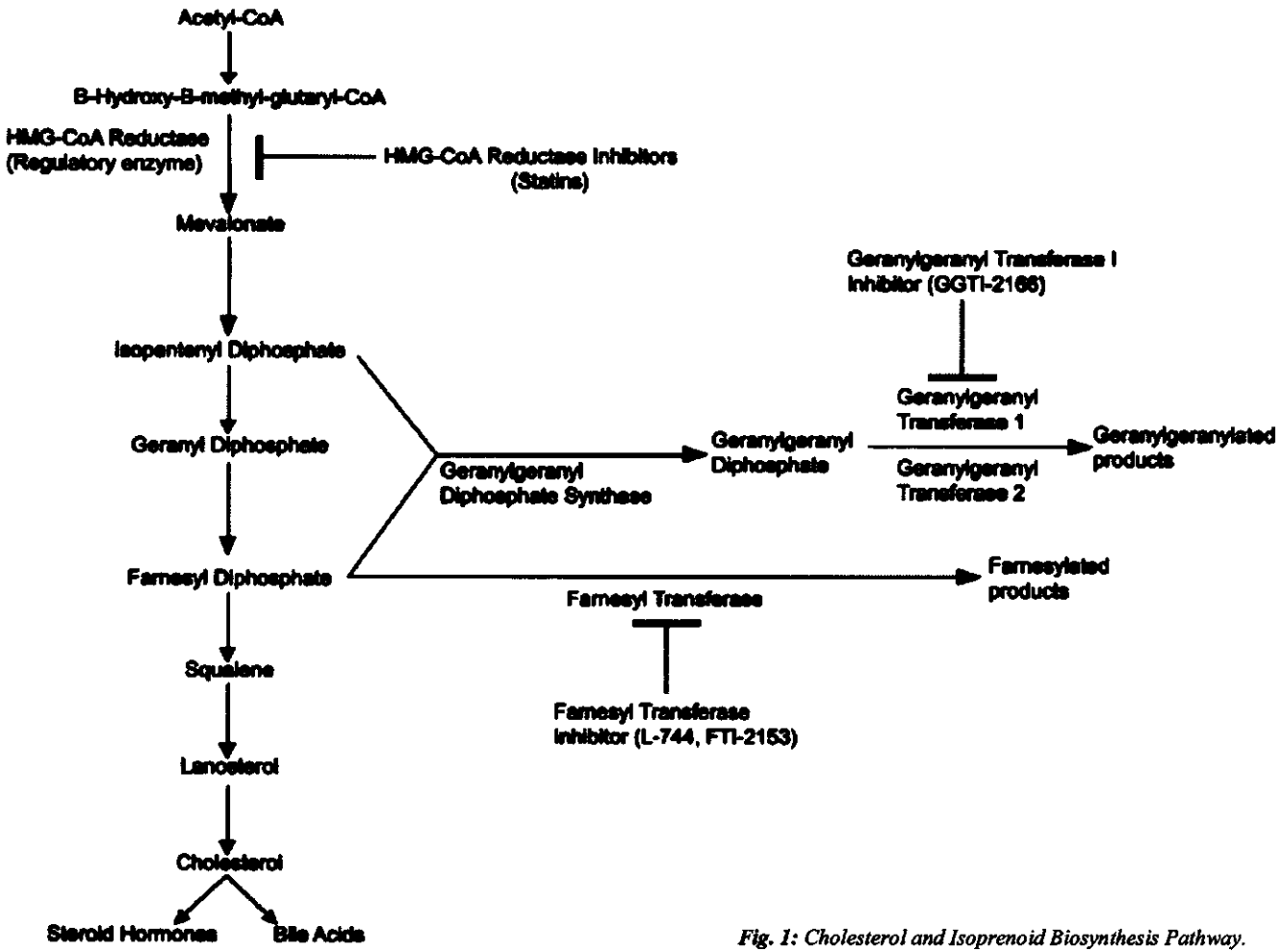

Fig. 1: Cholesterol and Isoprenoid Biosynthesis Pathway.

the migration of germ cells is disrupted, indicating that SDF-1a acts as a potent chemoattractant for zebrafish PGCs.

In the present study, we used statins, a class of potent competitive HMGCoAR inhibitors (Evans and Rees, 2002), to show that HMGCoAR activity is also required for vertebrate PGC migration. We found that the effect of statins could be overcome by injection of mevalonate but not squalene, indicating that cholesterol and its immediate precursors are not required for PGC migration. Further analysis of downstream components of the mevalonate pathway including the geranylgeranyl transferase 1 (GGT1), an enzyme responsible for the prenylation of proteins, suggests that geranylgeranylation is essential for correct PGC migration.

RESULTS

Cloning of Zebrafish HMGCoAR Orthologs

To examine a possible role of HMCCoAR in zebrafish PGC migration, we identified partial cDNAs of two HMGCoAR orthologs (hmgcr1 and hmgcr2) (Supplemental Figure S1 [http://www.developmentalcell.com/cgi/content/full/6/2/295/DC1]). Maternal hmgcr2 transcripts were evident in 4-cell stage embryos and uniform expression was observed during gastrulation, suggesting that the translated protein is present where PGCs migrate. hmgcr1 expression was observed in the liver and intestine of day 4 embryos and not at earlier stages.

HMGCoAR Is Required for Proper PGC Migration

To determine the effects of HMGCoAR during vertebrate development and PGC migration, we incubated early stage zebrafish embryos (2–4 hr post fertilization [hpf]) in media containing statins. Embryos treated overnight with either mevinolin (Lovastatin) or simvastatin (Zocor) exhibited developmental arrest, improper axis elongation, and compressed somites (Supplemental Figure S2). The developmental arrest and axis elongation phenotypes along with the EC_{50}s of these phenotypes were identical for both drugs. After mevinolin or simvastatin treatment, PGCs were widely dispersed, whereas untreated animals had a compact cluster of PGCs in the anterior yolk extension (data not shown). To demonstrate that the PGC migration phenotype represented a primary defect in this process and was not due to defects in somatic patterning (Supplemental Figure S2), embryos were treated with atorvastatin, the only statin that lacked a somatic phenotype in our assay (Figure 2).

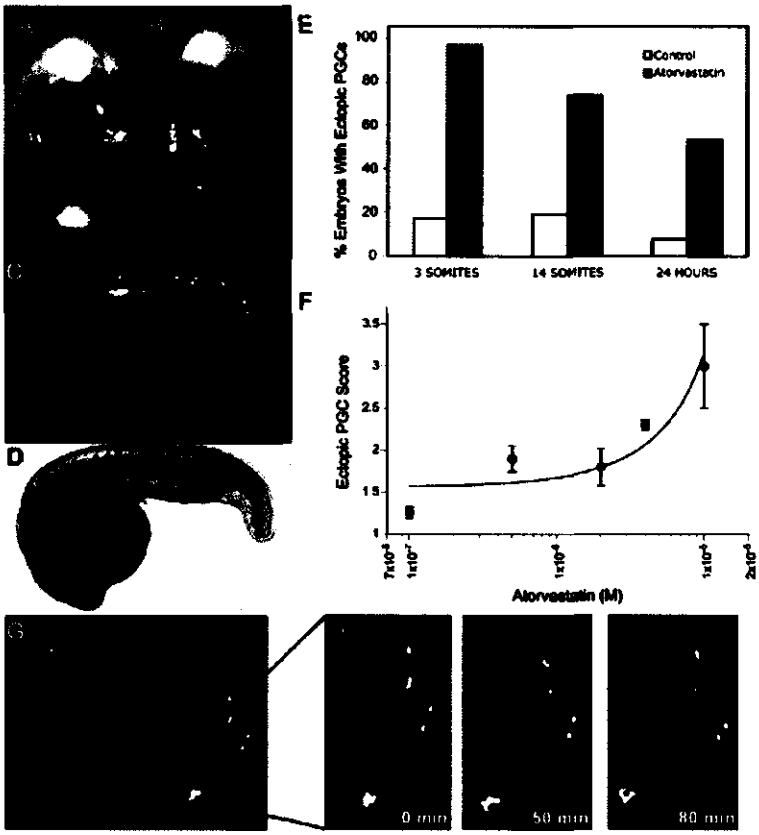

Figure 2. Migration of Primordial Germ Cells Is Altered by Atorvastatin Treatment
(A) A single fluorescent image from a time lapse (1-somite stage, first capture) of the movement of PGCs in a wild-type embryo injected at the 1-cell stage with gfp-nanos mRNA (1–2 nl, 60 ng/μl). (B) Embryos (1-somite stage) soaked in atorvastatin (10 μM) and injected with gfp-nanos mRNA exhibit ectopic PGCs that fail to cluster. (C) Later stage embryos (22-somite stage) exhibit many ectopic germ cells following atorvastatin (10 μM) treatment. (D) Brightfield image of an embryo (24 hpf) injected with gfp-nanos mRNAand treated with atorvastatin (10 μM) that exhibits only a mild tail defect. (E) Analysis of the effect of atorvastatin treatment on PGC migration at different developmental stages. The number of embryos at a given stage with more than two ectopic PGCs was determined and expressed as a percent of the total number of embryos examined (n = 64–84for each stage). (F) The effect of atorvastatin on numbers of ectopic PGCs is dose dependent. Embryos were soaked in atorvastatin (10 μM). At 24 hpf, embryos were each scored on the degree of ectopic PGCs (a level 1 embryo had a wild-type single gonadal cluster and a level 4 embryo had no discernable cluster). Data represent the mean ± SEM from three to four experiments/dose. (G) Initial image from a time-lapse movie of an atorvastatin-treated (10μM) embryo, taken at the 14-somite stage. (H) Three captured images from the time-lapse movie as in (G) taken at 1, 50, and 80 min after the 14-somite stage.

Atorvastatin treatment resulted in impaired PGC migration (Figures 2A–2C) without the severe morphological defects observed with the other statins (Figure 2D). Embryos treated with atorvastatin showed a PGC migration phenotype at the end of gastrulation, resulting in PGCs that failed to align along the anterior and lateral borders of the trunk mesoderm. By the 3-somite stage, over 90% of the embryos had ectopic

germ cells compared to less than 20% in the wild-type (Figure 2E). Furthermore, the effect of atorvastatin on PGC migration was dose dependent (EC_{50} 4 μM) (Figure 2F). As development proceeded, some of the ectopic PGCs eventually migrated to the correct location, but still, by 24 hr, more than 50% of the treated embryos had ectopic PGCs (compared to less than 10% in the wild-type) (Figure 2E). Time-lapse studies of atorvastatin-treated embryos indicate that GFP-labeled PGCs were motile (Figures 2G and 2H and Supplemental Movie S1). This suggests that inhibition of HMGCoAr activity leads to PGC migration defects either by affecting the speed of migration or by impairing directional migration.

Rescue of Statin-Induced Defects by Mevalonate

To examine the specificity of the statin-induced effects and provide evidence that the observed phenotype resulted from the inhibition of HMGCoAR, we injected embryos with mevalonate (the immediate product of HMGCoAR activity; see Figure 1) prior to statin treatment. Mevalonate injections resulted in a complete rescue of the PGC migration phenotype caused by all three statins (Figure 3C). As a control, embryos were injected with sodium citrate, a salt with a similar molecular weight as mevalonate, prior to statin treatment, and this failed to reverse the PGC phenotype (data not shown). Mevalonate not only reversed the PGC migration defects caused by statin treatment, but also fully rescued the somatic phenotype caused by mevinolin and simvastatin (Figure 3D). Thus, both PGC migration as well as embryonic patterning require components of the HMGCoAR pathway. Moreover, these data suggest that HMGCoAR activity does not provide directional information to PGCs since its product, mevalonate, can allow proper PGC migration even when it is uniformly applied.

Injections with Farnesol and Geranylgeraniol Rescue Atorvastatin Effects

Mevalonate is utilized to synthesize a variety of cellular compounds that include ubiquinones, carotenoids, isoprenoids, and cholesterol (Santos and Lehmann, 2004). To determine directly whether cholesterol or its derivatives played a role in zebrafish PGC migration, we tested squalene, an intermediate in the pathway specifically required for de novo cholesterol synthesis (Figure 1). Squalene injection failed to rescue the effects of statin treatment (data not shown), suggesting that cholesterol-independent compounds of the HMGCoAR pathway control PGC migration.

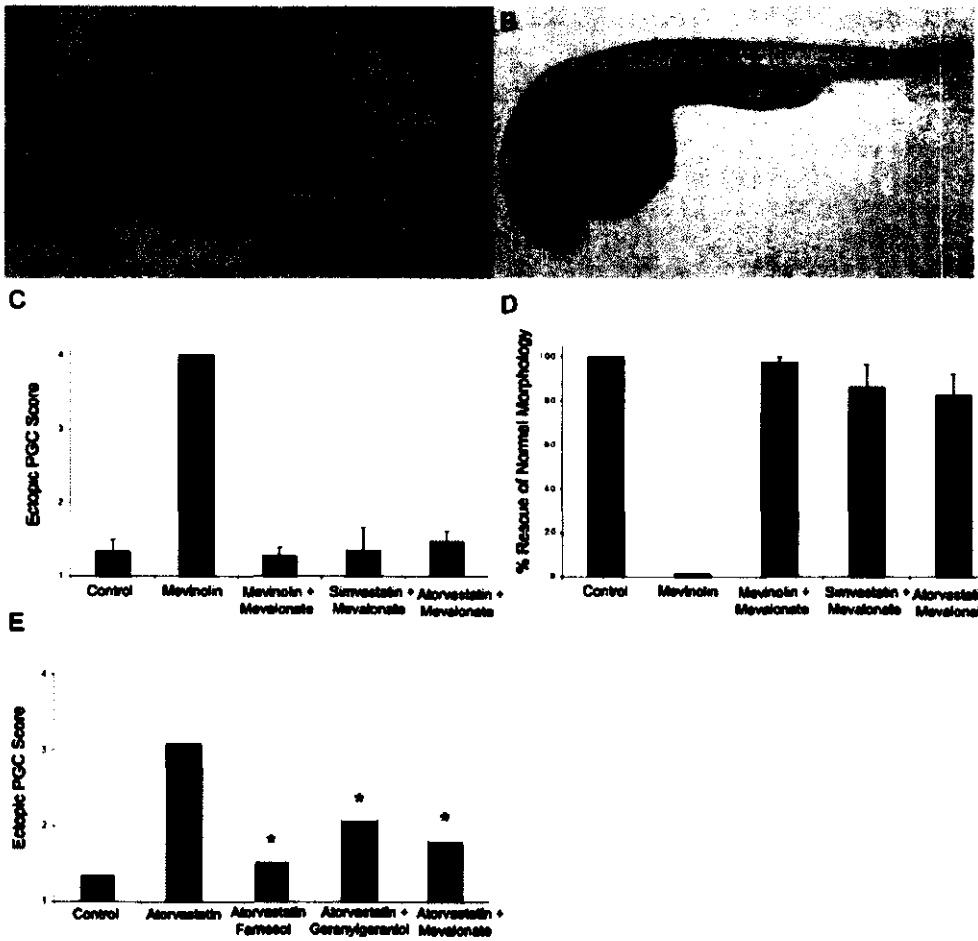

Fig. 3:. Injection of Isoprenoid Intermediates Abrogates the Effects of Statins
(A) Uninjected embryos show severe developmental defects after 24 hr of mevinolin (1.2 μM) treatment.
(B) Embryos injected at early cell stages (1–16 cell stage) with mevalonate (1–2 nl, 0.5 M) and then soaked in mevinolin (1.2 μM, 24 hr) exhibit normal morphology.
(C) The appearance of ectopic PGCs following statin treatment is prevented by mevalonate injections. Embryos at early cell stages were injected with gfp-nanos mRNA and mevalonate as in (B) and soaked overnight in statin drugs (mevinolin [1.2 μM], simvastatin [2.0 μM], and atorvastatin [10 μM]). At 24 hpf, the PGC score was determined. Data represent the mean ±SEM from three to four experiments.
(D) Mevalonate injection rescues the somatic defects observed in embryos treated with statins. Embryos at early cell stages were injected with mevalonate as in (B) and soaked overnight in statin drugs: mevinolin (1.2 μM), simvastatin (2.0 μM), and atorvastatin (10 μM). At 24 hpf, developmental defects were scored. Data represent the mean ±SEM from three to four experiments.
*(E) Embryos injected at the 1–16 cell stage with farnesol (1–2 nl, 1 M), geranylgeraniol (0.5–1 nl, 1 M) or mevalonate (1–2 nl, 0.5 M) and then soaked overnight with atorvastatin (10 μM) show normal PGC migration. Statistics were performed using ANOVA with a post hoc test that utilizes a Bonferroni correction. Data represent mean SEM, * $p<0.01$ difference from atorvastatin alone.*

To test whether the isoprenoid synthesis branch of the pathway (via geranylgeranyl transferases and farnesyl transferases) mediates the effect of statins on PGC migration, we injected embryos with compounds that would increase the cellular levels of the substrates of these enzymes before immersion in atovastatin (10 μM). Embryos injected with geranylgeraniol, an alcohol that is readily converted to geranylgeranyl diphosphate (Crick et al., 1994) prior to statin treatment exhibited normal PGC migration and were as equally protected as embryos injected with mevalonate (Figure 3E). Farnesol is an alcohol that can elevate cellular farnesyl diphosphate, a precursor required both by geranylgeranyl transferase and farnesyl transferase (Figure 1). Injection of farnesol also rescued the PGC migration defect of embryos from statin treatment (Figure 3E). Our results support the idea that the PGC migration defect induced by statins is the result of impaired geranylgeranylation of target proteins caused by reduction in cellular geranylgeranyl diphosphate (Figure 1). Furthermore, these results are consistent with genetic studies in *Drosophila* where mutations in geranylgeranyl diphosphate synthase and farnesyl diphosphate synthase, the enzymes that produce farnesyl diphosphate and geranylgeranyl diphosphate, respectively, result in germ cell migration defects (Santos and Lehmann, 2004).

Geranylgeranyl Transferase Activity Is Required for Correct PGC Migration

To examine whether geranylgeranyl transferase (GGT1) or farnesyl transferase activities influence PGC migration, embryos were treated with different doses of specific inhibitors and the position of PGCs was determined after 1 day of development. High doses of the selective farnesyl transferase inhibitor FTI-2153 (FTase IC_{50}, 1.4 nM over GGT I IC_{50}, 1700 nM) (Crespo et al. 2001 and Sun et al. 1999) had no effect on PGC migration (data not shown). A second farnesyl transferase inhibitor (L-744) (Kohl et al., 1995) also had no effect on PGC migration (data not shown). In contrast, injection of a selective inhibitor of GGT1 (GGTI-2166, 100-fold more selective at inhibiting Rap1A [IC_{50}, 300 nM] over farnesyl transferase as measured by H-Ras processing) (Sun et al., 1999) caused a strong PGC migration phenotype (Figures 4A–4D) and only mild morphological defects, which were manifested as a slight kink in the notochord (Figure 4D, arrowhead).

The effect of GGTI-2166 on PGC migration was dose dependent (approximate EC50 30 M) (Figure 4E). To determine the cellular basis for the GGTI-2166-induced abnormal cell migration, the behavior of GFP-labeled PGCs was analyzed by time-lapse microscopy (Supplemental Movies S2 and S3). The formation of cellular protrusions was normal in PGCs treated with GGTI-2166. However, a significant reduction in the migration speed in treated PGCs was observed (from 2.170 ± 0.155 to 1.515 ± 0.095 μm/min in GGT1-treated embryos $p < 0.001$). These data suggest that protein prenylation, specifically by GGT1, is required for correct PGC migration and that HMGCoAR activity is needed to provide GGT1 substrates.

To assess the potential role of GGT1 activity on PGC migration, we identified the zebrafish ortholog of this gene. Zebrafish GGT1 is approximately 70% identical to the human ortholog at the amino acid level (Supplemental Figure S3). Similar to

hmgcr2 expression, maternal ggt1 transcripts were evident in 4-cell stage embryos and uniform expression was observed during gastrulation (Supplemental Figures S1 and S3). These data suggest that GGT1 is present in positions where the PGCs migrate.

Do SDF-1 and HMGCoAR Act in the Same Pathway to Regulate PGC Migration?

Previously, it was shown that the G protein-coupled receptor CXCR4b and its ligand SDF-1a provide directional guidance cues to migrating PGCs. One possibility to explain the effect of statin treatment on PGC migration could be that HMGCoAR activity is required for the expression or activity of the SDF-1 ligand. We therefore analyzed the expression of sdf-1a in statin-treated embryos and found that atorvastatin treatment did not alter sdf-1a expression (Figures 4F and 4G). To further test if statins and the SDF-1a receptor CXCR4b function together or independently, we impaired both pathways by injecting atorvastatin and morpholino antisense oligonucleotides directed against cxcr4b (Heasman et al. 2000 and Nasevicius and Ekker 2000). Doses were selected to induce mild migration defects. The combined effect of atorvastatin and cxcr4b morpholino was not greater than the sum of each independent treatment (Figure 4H), indicating that HMGCoAR and SDF-1a function in distinct pathways.

DISCUSSION

The results of this study indicate that pharmacologic inhibition of protein prenylation, either through the inhibition of GGT1 directly or by limiting the supply of precursors for the reaction it catalyzes, impairs the migration of PGCs. The observation that correct PGC migration in zebrafish depends on HMGCoAR activity and isoprenylation is consistent with studies in *Drosophila* (Santos and Lehmann 2004 and Van Doren et al. 1998) and suggests that this pathway is evolutionarily conserved.

Understanding the mechanisms that regulate PGC migration is likely to help formulate a general model for long-range cell migration during development and disease. This is illustrated by the observation that the chemokine, SDF-1, and its receptor, CXCR4, provide guidance cues to direct PGCs to the developing gonad of mice and fish (Ara et al. 2003; Doitsidou et al. 2002 and Molyneaux et al. 2003) and that a receptor related to CXCR4 is required for PGC migration in *Drosophila* (Kunwar et al., 2003). Further, a number of reports indicate that SDF-1 plays a critical role in determining the destination of metastatic tumor cells (Muller et al., 2001) and the migration of other cell types during development (Peled et al., 1999).

The parallels between proteins that influence PGC migration and those that regulate cancer cell metastasis are further substantiated by our observation that inhibition of GGT1 results in altered PGC migration. Several studies find that statins or specific prenylation inhibitors impair the chemotactic migration of cancer cells (Morgan et al., 2003), the migration of human monocytes (Wong et al., 2001), and the migration of lymphocytes (Walters et al., 2002).

The present study utilizes highly specific enzyme inhibitors to identify likely regulators of PGC migration. This approach has a number of advantages in that multiple isoforms and not individual proteins can be targeted. Additionally, when

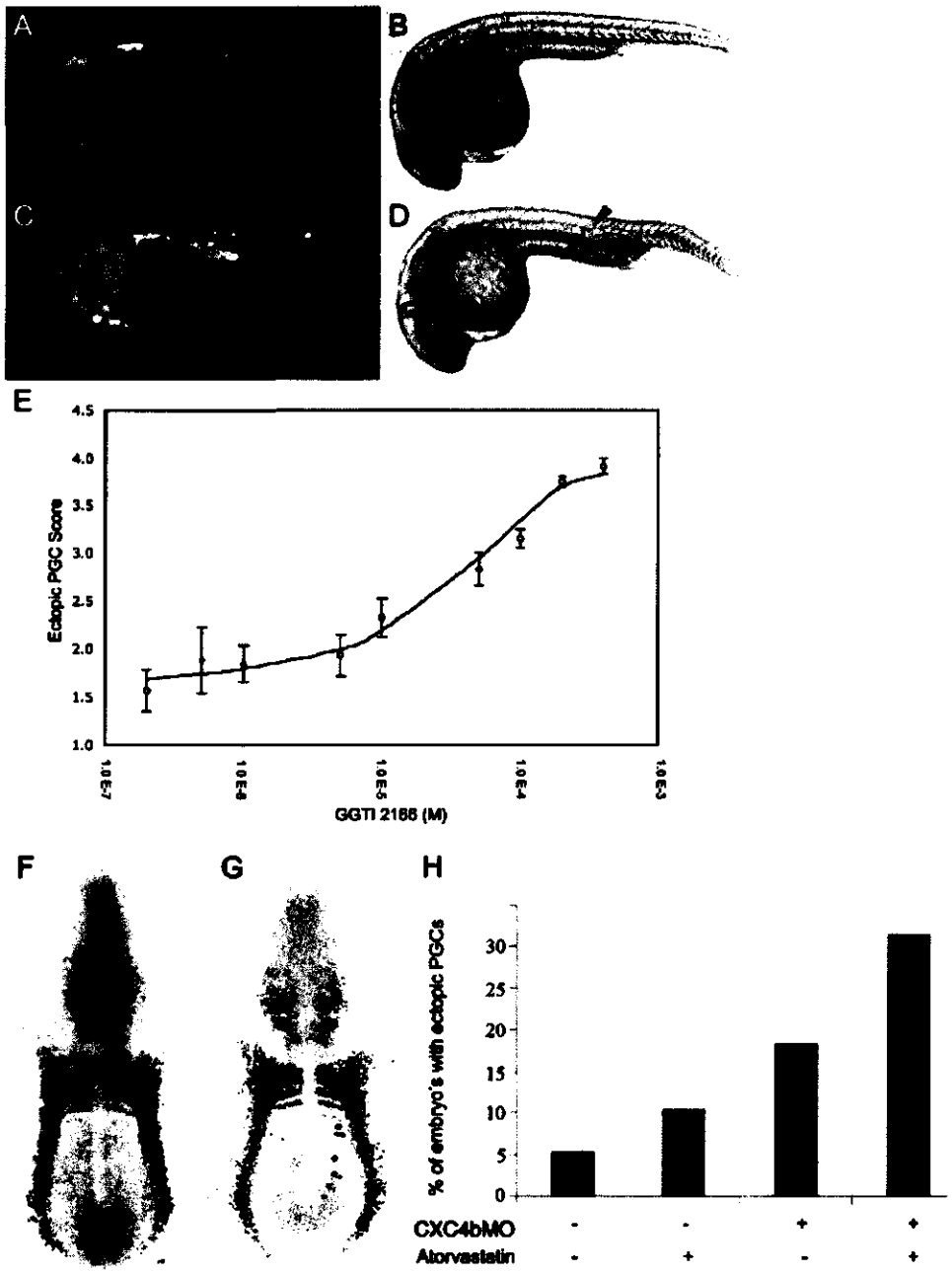

Fig. 4. (For figure legend, see next page)

genetic studies impair zygotic gene expression, residual maternally derived protein, which may be experimentally difficult if not impossible to eliminate, can mask the corresponding phenotype. Conversely, it can be difficult to establish that the phenotype resulting from soaking whole embryos in a pharmacologic reagent results from the specific inhibition of a protein or protein family. We addressed this issue by injecting embryos with mevalonate prior to their exposure to statins. This procedure rescued all phenotypes associated with statin treatment, supporting the notion that the observed effect is specific to the mevalonate pathway (Figure 3).

Although mevalonate rescued the effect of all the statins utilized in this study, the phenotypes induced by the different statins were not identical. Simvastatin and mevinolin produced more severe somatic phenotypes than atorvastatin, one of the most hydrophobic statins. One possibility is that atorvastatin partitions into the yolk, which may limit both the availability and concentration of the drug within the embryo.

Our observation that atorvastatin-induced PGC migration defects were rescued by both geranylgeraniol and farnesol points to a potential role for geranylgeranyl transferase or farnesyl transferase activities in this process. However, two farnesyl transferase inhibitors failed to show any effect on PGC migration, while a GGT1-specific inhibitor (Sun et al., 1999) demonstrated a role for geranylgeranylated rather than farnesylated proteins in the process. The fact that farnesol also rescued atorvastatin-induced migration defects could be due to the presence of enough cellular isopentenyl diphosphate (even after HMGCoA reductase inhibition) such that farnesyl diphospate can be converted to geranylgeranyl diphosphate by the action of geranylgeranyl diphosphate synthase (see Figure 1).

Despite the striking conservation for the role of HMGCoAR in *Drosophila* and zebrafish PGC migration, an important difference between the two systems should be noted. In *Drosophila*, the expression pattern of HMGCoAR can determine the direction of PGC migration. In zebrafish, however, the spatial distribution of HMGCoAR activity fails to provide the PGCs with directional cues since zebrafish HMGCoAR genes are not specifically expressed in regions toward which the PGC migrate (Supplemental Figure S1) and uniform application of mevalonate simply rescued rather than interfered with PGC migration. One possibility is that HMGCoAr activity merely

Fig. 4 (see previous page):. *GGT1 Activity Is Required for PGC Migration*
(A) A fluorescent image of a wild-type embryo (24 hpf) injected with gfp-nanos mRNA at the 1-cell stage reveals a cluster of PGCs.
(B) A brightfield image of the embryo in (A).
(C) A fluorescent image of an embryo (24 hpf) that was injected with gfp-nanos mRNA at the 1-cell stage and immediately soaked in the geranylgeranyl transferase inhibitor (GGTI-2166; 40 μM), resulting in ectopic migration of many PGCs.
(D) A brightfield image of the embryo in (G) showing a mild notochord defect (arrowhead).
(E) Dose response of GGTI-2166 on PGC migration defect. Data represent the mean ±SEM from three to six experiments for each dose. sdf-1a expression is not altered in atorvastatin-treated embryos. A two-color whole mount in situ hybridization experiment was performed using sdf-1a (brown) and nanos-1 (blue, marks germ cells) antisense riboprobes on wild-type (F) and atorvastatin-treated embryos (G).
(H) Embryos injected with a cxcr4b morpholino to inhibit translation of cxcr4b exhibited a PGC phenotype that is additive with atorvastatin treatment. All embryos were injected with gfp-nanos mRNA and a control morpholino (1–2 nl, 0.02 mM) or cxcr4b morpholino (1–2 nl, 0.02 mM) at the 1-cell stage. Half of the injected embryos from each group were immediately treated with atorvastatin (4.0 μM) or embryo medium.

provides a factor necessary for PGC migration, while directional cues are under the sole control of SDF-1a (Doitsidou et al., 2002). Alternatively, while isoprenylation itself may not be spatially controlled, the target of its function, i.e., the substrate for isoprenylation, may be expressed along the PGC migratory path and provide directional cues for PGCs independent of SDF-1.

Our data suggest that a mevalonate-dependent prenylation reaction mediated by GGT1 is important for correct PGC migration. It has been proposed that protein prenylation mediates more than just the association of a protein to the membrane, but can act as a specific mediator of protein-protein interactions (Sinensky, 2000). Our results, together with those of Santos and Lehmann (2004), further suggest that there are yet-to-be-discovered proteins whose prenylation is essential for correct PGC migration as part of a highly conserved signaling pathway.

EXPERIMENTAL PROCEDURES

Zebrafish

Methods for breeding and raising zebrafish were followed as described (Westerfield, 1995). Embryos were obtained from natural matings of wild-type (Oregon, AB) fish and staged according to criteria previously outlined (Kimmel et al., 1995) and by hours postfertilization (hpf).

Statin Pharmacology

To inhibit HMGCoAR activity, embryos were treated with statins, competitive inhibitors of HMGCoAR. Embryos were incubated overnight at 28°C in petri dishes (35 × 10 mm) containing mevinolin (Sigma Chemical), simvastatin (LKT Laboratories), and atorvastatin (LKT Laboratories) in embryo medium (EM) (Westerfield, 1995).

Statistical Analysis of Atorvastatin Phenotype

Embryos were treated with atorvastatin (10 μM) and then fixed at a variety of stages and subject to two-color in situ hybridization using *sdf*-1a and nanos riboprobes (Doitsidou et al. 2002 and Koprunner et al. 2001). The ratio of the number of embryos with more than two ectopic PGCs to the total number of assayed embryos was determined.

Microinjections

Embryos were injected with glass microelectrodes fitted to a gas pressure injector (PLI-100, Harvard Apparatus, Cambridge, MA). Electrodes were pulled (P-97, Flamining/Brown) and filled with a stock solution of mevalonate (500 mM, Mevalonolactone, Sigma Chemical), sodium citrate (500 mM, FischerBiotech), farnesol (1 M, Sigma Chemical), geraynlgeraniol (1 M, Sigma Chemical) and/or gfp-nanos mRNA. Phenol red solution (0.2% final concentration) was added to all injection solutions to visualize injected embryos. Typically, embryos were initially injected with gfp-nanos mRNA and then sorted into groups that were subject to a second injection of mevalonate or buffer.

Farnesyl and Geranylgeranyl Transferase Inhibitors

Ras farnesyltransferase inhibitor (L744; Sigma) was made up to a final concentration of 4 mM. 1-cell stage embryos were injected with 1–2 nl of farnesyltransferase inhibitor and 1–2 nl of gfp-nanos mRNA. Some embryos were also soaked in farnesyltransferase inhibitor overnight. GGT1 inhibitor GGTI-2166 (Mol. Wt. 434.53) and farnsyl transferase inhibitor FTI-2153 (Mol. Wt. 539) were resuspended in DMSO (50 mM).

Microscopy

Embryos were anesthetized (Tricaine, 170 μg/ml) and placed on depression slides. Images were taken on a Zeiss Axio Vision 3.0 Camera mounted on a Leica MZFL-III stereomicroscope. Some images were captured using a Zeiss Axio Vision 3.0 camera mounted on a Zeiss Axioplan II microscope.

ACKNOWLEDGEMENTS

The authors want to thank Andrew Hamilton and Said M. Sebti for kindly providing inhibitors (GGTI-2166 and FTI-2153) and technical advice. We also want to thank Evelyn Santana for helpful suggestions. This work was supported in part by US NIH grant R01 DK060369 and by a Pew's Scholar's Award to S.A.F. E.R. is supported by grants from Volkswagen-Stiftung and the DFG.

Chapter 4

Discussion

Discussion

G-protein coupled receptors and PGC migration

Guided cell migration is important for a wide variety of processes in embryonic development, homeostasis and disease. To reach their targets, the migrating cells rely on positional information that is often provided in the form of a unique spatial distribution of secreted molecules that function as repellents or attractants. Over the last few years, considerable progress has been made in identifying the molecular nature of both extracellular and intracellular components involved in guided cell migration. One particularly important family of molecules implicated in this process is the family of G-protein coupled receptors that recognise guidance cues.

In vertebrates, a central group of G-protein coupled receptors -the chemokine receptors - is involved in guided migration particularly in the immune and nervous systems (Bajetto et al., 2002; Hasegawa and Fujita, 2001). A classical example falling into this category is cell guidance by the chemokine SDF-1 and its G-protein-coupled receptor CXCR4 (Kucia et al., 2004). SDF-1 is considered to be the only agonist for CXCR4, and CXCR4 appears to be the only receptor for SDF-1. Consistently, SDF-1 or CXCR4 mutant mice exhibit very similar phenotypes including defective B-cell lymphopoiesis, abnormal bone-marrow myelopoiesis as well as cell-migration defects in the cerebellum (Ma et al., 1998; Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998).

In chapter 2 of this thesis, the involvement of SDF1/CXCR4 signalling in directing PGC migration in zebrafish was described. We have shown a remarkable correlation between *sdf*1a RNA expression domains and the location of the PGCs that express the receptor CXCR4b. Furthermore, the dynamic changes in *sdf*1a expression pattern prefigure the migration route of the PGCs. Knocking down the activity of SDF-1a or its receptor *cxcr*4b resulted in severe migration defects demonstrating that these two molecules are indispensable for directional PGC migration. Last, ectopic expression of SDF1a was sufficient to attract PGCs to these locations, demonstrating that SDF1a is a necessary and sufficient instructive molecule guiding zebrafish PGC migration. This finding reflects a remarkable conservation in the molecular mechanisms that govern migration of various cell types in different organisms (e.g., primordial germ cells in zebrafish and leukocytes in mammals).

The involvement of CXCR4b in zebrafish PGC migration raised the possibility that the mechanism of PGC guidance could be conserved in other organisms, as well. Indeed, a role for CXCR4 and its ligand has been demonstrated in PGC migration in mouse (Ara et al., 2003; Molyneaux et al., 2003). Nevertheless, in contrast to the findings in zebrafish where SDF1/CXCR4 signalling appears to be required for all stages of migration, in mouse it is only the last step of PGC migration (gonad colonization) that appears to be dependent on CXCR4 function, raising the possibility

that additional signalling pathways might be involved in guiding the cells at earlier stages. Another difference regarding the role of CXCR4 between mouse and zebrafish is that in contrast to the findings in zebrafish, in mice knocked out for either CXCR4 or SDF1 the number of PGCs is reduced (Ara et al., 2003), implicating this pathway in supporting cell survival in this organism.

Another intriguing case where the role of CXCR4/SDF1 pathway in guiding PGC migration proved to be conserved is in the case of chick. In this organism the PGCs use the vascular system as a vehicle to transport themselves to the region of the gonad, where they exit the blood vessels by penetrating the gonadal epithelium (Niewkoop and Sutasurya, 1979). This route of PGC migration is noticeably reminiscent of leukocyte trafficking, where the cells migrate into and out of the blood vessels to arrive at their target. It was recently shown that chick PGCs, just like leukocytes, are guided by SDF1 signalling to leave the blood circulation at the right region and populate the gonad (Stebler et al., 2004).

Although chemokines have not been identified in the genome of *Drosophila*, a seven-transmembrane receptor encoded by the gene trapped in endoderm (trel) is required for the migration of the PGCs across the midgut epithelium to the gonadal mesoderm (Kunwar et al., 2003). This finding suggests that the conservation of the mechanisms regulating PGC migration can be extended to *Drosophila* as well.

Involvement of the isoprenoid pathway in PGC migration

Genetic screens in *Drosophila* identified some of the first genes involved in PGC migration. The gene *columbus*, encoding for the enzyme HMGCoAR, was shown to mediate attraction for PGCs (Van Doren et al., 1998). This enzyme catalyzes the production of mevalonate in the cholesterol/isoprenoid biosynthesis pathway and is located intracellularly. Therefore, it was considered that HMGCoAR does not act as the actual attractant for PGCs but rather, contributes to the production of another molecule that acts as an attractant.

To examine the possible evolutionary conservation of the above pathway from *Drosophila* to higher organisms, in chapter 3 of this thesis the role of HMGCoAR in zebrafish PGC migration was analysed. In this work, a pharmacological approach was employed to block the activity of the protein of interest. This approach, despite its inherent disadvantage (e.g., possible side effects of the drugs in use) provides a very powerful tool in comparison to more conventional genetic methods that result in the loss of function of specific genes (e.g., genetic mutations, inhibition of mRNA translation by antisense oligonucleotides, etc). An advantage of using small chemical compounds lies in their ability to interfere with protein activity, thereby targeting also maternally provided proteins whose function cannot be eliminated using antisense oligonucleotides. Additionally, utilizing this approach, it is possible to inhibit several isoforms of a protein, if such exist, a task that cannot be achieved when inhibiting the function of a single gene product genetically. Zebrafish is an ideal model organism for this approach as it is possible to provide drugs simply by providing them in the water in which the embryo develops.

Indeed, whereas using modified antisense oligonucleotides to knock down HMGCoAR function had no effect on PGC migration, the pharmacological approach proved to be fruitful. Using different statins that are known to inhibit HMGCoAR activity we could demonstrate a requirement for this enzyme in PGC migration as in treated embryos a significant fraction of the PGCs fail to arrive at their target and were therefore found in ectopic positions. Furthermore, the migration speed of PGCs was found to be significantly lower in treated embryos, which could be the underlying reason for the observed phenotype.

Although HMGCoAR is required for proper PGC migration in zebrafish, in contrast with its role in *Drosophila,* its function is unlikely to be involved in the production of an attractant. Two lines of evidence support this idea. First, the known zebrafish HMGCoAR genes are either ubiquitously expressed or not expressed during the time PGCs migrate (results in chapter 3) and therefore they are not likely to provide PGCs with directional cues. Second, uniform application of mevalonate, the downstream product of HMGCoAR activity, not only did not interfere with PGC migration but also completely rescued the statin phenotype (Fig. 3C in chapter 3). This result raises the possibility that HMGCoAR plays a permissive role for PGC migration by contributing to optimal cell motility. Its activity could be required either in the PGCs themselves, or in their environment to modify a factor that is important for proper PGC migration.

Using the pharmacological approach, it was possible to further dissect the pathways downstream of mevalonate production and to identify downstream components involved in PGC migration. This analysis revealed that squalene, an intermediate component of the pathway that is required for cholesterol synthesis (see Fig.1 in chapter 3), could not rescue the phenotype induced by statins. Therefore we could exclude the possibility that the effect of statins on PGC migration resulted from reduced cholesterol synthesis. Furthermore, we showed that one enzyme in the isoprenoid branch of the mevalonate pathway, geranylgeranyl transferase 1 (GGT1), is involved in PGC migration downsream of HMGCoAR. Interestingly, at the same time this study was performed, it was shown that *Drosophila* embryos mutated for the β-subunit of GGT1 display germ cell migration defects that are similar to the ones observed for *hmgcr* mutant flies (Santos and Lehmann, 2004b).

Geranylgeranyl transferase 1 (GGT1) together with farnesyl transferase (FT) and geranylgeranyl transferase 2 (GGT2) belongs to the group of prenyltransferases that catalyse the attachment of prenyl lipid anchors to target proteins (protein prenylation). Prenylation is a common posttranslational modification that can direct the subcellular localization of proteins and influence protein-protein interactions (Maurer-Stroh et al., 2003). Inhibition of prenyltransferases has been reported in the past to impair migration of several cell types (Greenwood et al., 2003; Kusama et al., 2003; Sawada et al., 2002; Virtanen et al., 2002; Walters et al., 2002; Wong et al., 2001).

The identified role of GGT1 in PCG migration narrows down the possible mode of action of the isoprenoid pathway in this process, pointing to protein geranylation as an important requirement for optimal cell motility. The main question that remains open is, what are the targets of GGT1 that are relevant for PGC migration. The possibility that GGT1 directly modifies SDF1a or CXCR4b or that it acts downstream to them seems unlikely; first, neither of these molecules contains sequence motifs that are known to be recognized by GGT1. Second, the two pathways appear to control

different cell behaviours – whereas SDF1a and CXCR4b are crucial for directed migration, the isoprenoid pathway appears to impair only the migration speed, but not directionality. Consistently, no synergistic genetic interactions could be observed between the two pathways (Fig. 4H, in chapter 3).

An important point for investigation that would facilitate the identification of GGT1 targets is whether GGT1 activity is required in the PGCs themselves or rather in their environment. Inhibitors of the isoprenoid pathway have been reported to interfere with cell migration and chemotaxis by acting on both, the migrating cells e.g., (Kusama et al., 2003; Wong et al., 2001) and the target tissue (Cornell et al., 1995; Santos and Lehmann, 2004b).

The formation of SDF1a gradient

Migratory cells are able to sense extracellular signals and adjust their movement in response to the concentration gradient of these molecules. One of the requirements for a proper chemotactic response is therefore the formation of a stable gradient of the chemoattractant.

In the work described in this thesis, the distribution of the attractive cue was monitored by following the expression of *sdf*1a RNA. However, the spatial distribution of the SDF1a protein in zebrafish has not been studied and the mechanisms underlying the formation of the putative SDF1a gradient are not known. A definitive answer to these questions would require the generation of specific antibodies that would allow monitoring SDF1a protein. Nevertheless, it is possible to approach this issue indirectly, utilizing the behaviour of the PGCs as an indicator for the spatial distribution of SDF1a. For example, information regarding the diffusion range of SDF1a was obtained with the analysis of PGC migration in *spadetail* mutant embryos. In these mutants, PGCs can occasionally arrive at a region located between two *sdf*1a RNA expressing domains. From this position, the cells are able to move towards either of the *sdf*1a RNA expressing domains (see Fig. 2D). This observation led to the conclusion that SDF-1a protein can most likely diffuse at least few cell diameters away from cells that produce its RNA.

In wild type embryos however, a very tight correlation between the position of the PGCs and *sdf*1a RNA expression is observed (Fig. 1, chapter 2). Perhaps the most striking example of this phenomenon can be observed during a step when the PGCs move posteriorly as a cluster (step depicted in Fig. 1, E-F, chapter 1). During this time, the expression domain of *sdf*1a gradually recedes to the posterior (Fig. 1, K-L, chapter 2) and PGCs appear to respond to this shift by following the front of its receding expression (Fig. 1, O, chapter 2). It is therefore possible that SDF1a functions by providing the PGCs with an 'attractive path' rather than acting as a long-range signal. Along this attractive path SDF1a gradient could be established as a combined result of transcriptional regulation and fast RNA turnover. Such a mechanism has been recently demonstrated to regulate the establishment of the FGF8 gradient during chicken and mouse posterior patterning (Dubrulle and Pourquie, 2004).

Studies on morphogens showed that gradients are not merely formed by free diffusion of a secreted factor. Rather, components of the extracellular matrix are

involved in establishing a gradient and modifying the biological properties of the secreted molecule (Lander and Selleck, 2000; Perrimon and Bernfield, 2000). For example, the spatial distribution of Dpp in *Drosophila* is known to depend on its reversible binding to heparan sulfate proteoglycans, an interaction that possibly restricts the difusion of this morphogen closer to the cell surface and at the same time mediates its spreading from one cell to another (Belenkaya et al., 2004). A similar interaction with HSPG has been reported in the case of mouse SDF1a (Amara et al., 1999). *In vitro* studies showed that that the attraction of haematopoietic progenitor cells mediated by SDF1 depends on binding to HSPG. This interaction optimized the presentation SDF1 to its receptor and at the same time facilitated the formation of a stable chemotactic gradient (Netelenbos et al., 2003; Netelenbos et al., 2002). It would be very interesting to investigate whether these observations are relevant *in vivo* in the case of the formation of SDF1a gradient in zebrafish. This issue could be addressed by knocking down the enzymes that are involved in the synthesis of such polysaccharides (e.g., heparan sulfotransferases) and observing the distribution of SDF1a protein as well as the PGC migratory behaviour in embryos knocked down for this molecule.

An intriguing complication regarding the formation of a chemotactic gradient that guides PGCs in the zebrafish embryo is that a second gene encoding for SDF1 was identified. This protein, SDF-1b, shows high level of sequence identity to SDF-1a and is able to attract PGCs when overexpressed to ectopic positions (Knaut et al., 2003). The spatial distribution of *sdf*1b RNA only partially overlaps with that of the *sdf*1a, and in general it is not expressed in regions towards which the PGCs migrate. Nevertheless, despite the fact that, during their migration, PGCs are often found in the vicinity of *sdf*1b expressing domains, they do not move towards these domains but rather remain in the route defined by the expression of *sdf*1a. An interesting issue for future investigation raised by this scenario is why do PGCs preferentially follow *sdf*1a expression in the wild type embryo. Answering this question could contribute to our understanding of how can cells that are able to perceive multiple and some times opposing signals, navigate accurately in a complex field of chemoattractants. .

Aspects of Cellular Behaviour: Motility versus Directionality

A major advantage of studying cell migration in zebrafish is the optical clarity of the embryos that facilitates high quality *in vivo* data acquisition using relatively simple microscopy. By labelling PGCs with GFP the behaviour of these cells could be observed in the intact organism (Knaut et al., 2002; Weidinger et al., 2002). In these studies it was demonstrated that PGCs show morphological features characteristic of motile cells, including cell polarization and formation of dynamic cellular extensions. In the present study, the behaviour of PGCs was studied in embryos in which SDF1a signalling was altered and compared with that observed in wild-type embryos.

Interestingly, the ability of zebrafish PGCs to extent protrusions and move was found to be independent of the chemoattracting signal. In embryos in which the SDF1a or CXCR4b activities were knocked down, PGCs lost their directionality but were nevertheless highly motile (Fig. 4, A-C, chapter 2). This cellular behavior is strikingly different from that described for neutrophils in which the chemotactic signal is crucial

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

Observing the cells at higher resolution (Fig. 5, chapter 2) revealed that PGCs in wild type embryos are often polarized and extend protrusions in the direction of the movement (polarized migratory behaviour). In contrast, in embryos in which SDF1 signalling was knocked down, PGCs often exhibited different cell behaviour, showing lack of polarity and extending protrusions to all directions (apolar stationary behaviour). Recently, in a more detailed analysis of PGC cellular behaviour during their migration, it was shown that PCGs alternate between these migratory and stationary behaviours, independently of SDF1a signalling (Reichman-Fried et al., 2004). However, although PGCs displayed a polarized migratory behaviour in the absence of SDF1a signalling, this phase was significantly shorter in comparison to the wild type situation. These data suggest that motility and polarization are intrinsic properties of PGCs that are independent of the guidance cue. SDF1a signalling provides the migrating cells with direction as well as it allows them to maintain their polarity for a longer time.

Another interesting observation regarding PGC behaviour is that, when SDF1a is overexpressed in these cells (which presumably creates a uniform high concentration of the chemoatrractant around them), they arrest their migration (Fig. 4, D, chapter 2). This is also observed in wild type embryos in regions where high levels of SDF1a RNA were present (Reichman-Fried et al., 2004). Such regions are the intermediate for PGC targets (e.g., the clustering point) and their final targets (the region where the gonad forms). A close examination of PGCs in regions where they encounter a uniform SDF1a concentration revealed that these cells extent protrusions in all directions but do not move relative to the somatic tissues. This is a very important feature of PGC behaviour that allows their close association with the tissues that express the highest levels of SDF1a and retains them in their intermediate and final targets.

The observations of PGC migratory behaviour prompt basic questions regarding the molecular mechanisms responsible for establishing cell polarity and for translating a chemical gradient into directed migration. In many migrating cells, downstream to G proteins are proteins of the phosphoinositide 3-kinase (PI3K) family. A significant insight into the importance of PI3K in controlling directional cell migration and cell polarity has come from studies carried out in *Dictyostelium* discoideum and neutrophils. In these studies, activation of PI3Ks results in the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) - at the leading edge of migrating cells in response to receptor activation and G-protein signaling (Chung et al., 2001; Devreotes and Janetopoulos, 2003; Iijima et al., 2002; Merlot and Firtel, 2003; Parent, 2004). The resulting asymmetric localization of PIP3 serves to recruit proteins that contain the pleckstrin homology (PH) domain to the leading edge, and these proteins are thought to activate downstream responses such as actin polymerization and, ultimately, the establishment of cellular polarity (Chen et al., 2003; Lemmon et al., 2002; Parent, 2004).

In this context, it was shown that in zebrafish, downstream of CXCR4b, PGC directional migration depends on the function of Gi proteins (Dumstrei et al., 2004). However, unlike neutrophils and *Dictyostelium*, cell polarity and directional migration are not correlated with polarized distribution of PIP3 on the cell membrane. Nevertheless, the PI3K pathway was found to be important for PGC motility and

morphology as the migration speed was reduced and the cell morphology was abnormal in cells in which this pathway is inhibited (Dumstrei et al., 2004).

Concluding remarks

The present study revealed two pathways involved in PGC migration in zebrafish. The isoprenoid pathway, which is required for optimal cell motility and the SDF1a/ CXCR4b pathway that provides the PGCs with directional information. Both pathways have been implicated in the regulation of cell migration in numerous developmental processes and also in human diseases. Therefore, the study of PGC migration in zebrafish can serve as a general model for understanding the principles of directed cell movement in development as well as in pathological conditions that result from aberrant cell migration.

Although the developmental aspects of PGC migration in zebrafish are now better understood, relatively little is known regarding the mechanisms that promote motility and directional migration of PGCs at the cell biology level. Further studies aimed at identifying the downstream components of SDF1a/CXCR4b pathway and unravelling the molecular mechanisms that control cell polarity and the architecture of the cytoskeleton would enhance our understanding of PGC migration and of processes in normal and abnormal development that operate using the same biological principles.

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Samenvatting

Primordiale geslachtscellen (PGCs) worden in een vroeg stadium van de embryogenese gevormd. In veel zich seksueel voortplantende dieren worden deze PCGs op een andere plaats gevormd dan de gonaden (geslachtsklieren). Daarom moeten deze PCG's naar de geslachtsklieren migreren om voor de productie van de uiteindelijke geslachtscellen te zorgen. Het onderzoek dat in dit proefschrift beschreven staat, had tot doel het moleculaire mechanisme te ontrafelen dat deze PCG's leidt van de plaats waar ze gevormd worden naar de gonaden. Dit moleculaire mechanisme werd bestudeerd in zebravis, een belangrijk modelorganisme voor vertebraten. Zebravis-embryo's ontwikkelen zich buiten het lichaam van de moeder en het embryo is transparant. Deze eigenschappen maakten het mogelijk om de migratie van PGCs efficiënt en gedetailleerd te bestuderen.

Andere studies hadden laten zien dat PCGs op vier verschillende posities in het zebravis-embryo gevormd worden. Van daar volgen zij een specifiek migratiepad om uiteindelijk aan het eind van de eerste levensdag de eindbestemming te bereiken. Gedurende deze migratietocht ontvangen de PCG's informatie van de somatische weefsels die ze passeren en die bepaalt het pad dat ze zullen volgen.

Het onderzoek beschreven in dit proefschrift had tot doel de moleculen te identificeren die deze gidsfunctie hebben. De gidsmoleculen werden geïdentificeerd m.b.v. een grootschalig antisense oligonucleotide experiment. Hierbij werden een chemokine receptor en het bijbehorende ligand geïdentificeerd als sleutelmoleculen. De receptor is genaamd CXCR4b en het ligand hiervan is het chemokine SDF1a. Van vergelijkbare ligand-receptor paren was eerder in andere dieren aangetoond dat zij het migratiepad van cellen reguleerden. Dit betreft migratie van cellen in verschillende ontwikkelingsprocessen en ziektes, zoals het gidsen van leukocyten naar de plaats waar een ontsteking is of van uitzaaiende kankercellen naar locaties waar secundaire tumoren gevormd kunnen worden.

De receptor CXCR4 wordt gemaakt door de PCGs en het ligand wordt uitgescheiden door de cellen van de weefsels die het migratiepad vormen. Door de functie van receptor of ligand uit te schakelen kon worden aangetoond dat beide essentieel zijn voor de PCGs om naar het einddoel te migreren. Verder kon een ander migratiepad gecreëerd worden door het ligand, SDF1a, door andere weefsels te laten maken. Dit laatste onderstreept de gidsfunctie van dit cytokine. De rol van een vergelijkbaar receptor-ligand paar in het begeleiden van PCGs op hun tocht naar de cellen die de geslachtsklieren zullen vormen, is nu ook aangetoond in muis en kip. Dit geeft aan dat het gidsmechanisme dat wij in zebravis-embryo's hebben ontdekt waarschijnlijk algemeen voorkomt bij vertebraten.

In Drosophila waren andere gidsmoleculen geïdentificeerd die een rol spelen bij PCG-migratie. Deze moleculen worden gevormd door de cholesterol/isoprenoid biosyntheseroute. Om te bepalen of deze biosyntheseroute ook een rol speelt bij de PCG-migratie in de zebravis, is gebruik gemaakt van moleculen die specifiek stappen in deze biosyntheseroute blokkeren. Vervolgens is getoetst of dergelijke blokkades de PCG-migratie beïnvloeden. Op deze wijze werd aangetoond dat remming van

HMGCoA-reductase (katalyseert een beperkende stap in de cholesterol-biosynthese) de snelheid van de PCG-migratie verlaagt. Daardoor bereiken de PCG's vaak abnormale posities in het embryo. Verdere analyses lieten zien dat Geranylgeranyl transferase 1 (GGT1), dat na HMGCoA reductase actief is in de biosyntheseroute, ook essentieel is voor een optimale PCG-migratie. Dit betekent dat moleculen die gevormd worden na de Geranylgeranyl transferase stap betrokken kunnen zijn bij PCG-migratie.

Dankwoord

Now that my thesis is completed I take the opportunity to go back in time and re-evaluate the three years of my PhD. I realize that my attainments during this time are to a great extent owed to the support and contribution of many people, in and out of the lab. To these people I would like to express my appreciation and gratitude.

First of all I would like to thank my supervisor and head of the group, Erez Raz, for trusting me and supporting me during all my steps, from the period I was establishing myself in his lab, to the time I was considering my future directions. Being in his lab was a unique educational experience.

The period working on SDF1 was incredibly intense and busy, and without the help of Julia I probably wouldn't have made it. Julia, your effort was invaluable to me and I am sorry for all the nightmares that disturbed your sleep during that time. Michal, thank you for all the interesting discussions, for teaching me time lapse microscopy, but most of all for your practical and emotional support when I mostly needed it. This lab was more than a working place for me; it felt most of the time like a family. Krasimir, Karin, Juerg, Heiko, Sonia, Natalia, Markus, and Elena, thank you for being more than colleagues to me.

Even more, I would like to thank my family; ευχαριστω τους γονεις μου για τον αθλο τους να μεταμορφωσουν μια "κακομαθημενη" σε επιστημονα... Επισης για την ανεξαντλητη συμπαρασταση τους αλλα και την κατανοηση τους για την αποφαση μου να ζησω μακρια απο την Ελλαδα.

I would also like to thank my 'Doctor-father' Prof. Ton Bisseling for his kind support; Prof. Just Vlak for his recommendations. Finally I would like to express my sincere gratitude to Rommert van den Bos who literally changed the course of my career by making it possible for me to enrol to the Wageningen University in the beginning of 2000.

And at last, my dear friends back in Greece and here in Goettingen who filled my life with warmth during this period and especially Pavlo, Bei, Alex, Lila, Alexandra, Olga, Francesca, Elpida, and Petro. Thank you for caring and forgive me if I neglected you in my busy times.

Goettingen, 09/01/2005

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Curriculum Vitae

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