Transition from non-motile behaviour to directed migration during early PGC development in zebrafish

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Summary

The migration of zebrafish primordial germ cells (PGCs) is directed by SDF-1a and serves as a model for long-range chemokine-guided cell migration. Whereas the development and migration of zebrafish PGCs have been studied in great detail starting at mid-gastrulation stages when the cells exhibit guided active migration [7-8 hours post fertilization (hpf)], earlier stages have not yet been examined. Here we show that the PGCs acquire competence to respond to the chemokine following discrete maturation steps. Using the promoter of the novel gene *askopos* and RNA elements of *nanos*1 to drive GFP expression in PGCs, we found that immediately after their specification (about 3 hpf) PGCs exhibit simple cell shape. This stage is followed by a phase at which the cells assume complex morphology yet they neither change their position nor do they respond to SDF-1a. During the third phase, a transition into a 'migratory stage' occurs as PGCs become responsive to directional cues provided by somatic cells secreting the chemokine SDF-1a. This transition depends on zygotic transcription and on the function of the RNA-binding protein Dead end and is correlated with down regulation of the cell adhesion molecule E-cadherin. These distinctive morphological and molecular alterations could represent a general occurrence in similar processes critical for development and disease.

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Introduction

In many organisms, primordial germ cells (PGCs) are specified early in development and subsequently migrate towards the region of the gonad where they give rise to gametes (Molyneaux and Wylie, 2004; Raz, 2004; Santos and Lehmann, 2004). We study PGC development in zebrafish, a vertebrate model organism whose germline is specified through the inheritance of asymmetrically localized maternally provided determinants, collectively termed germ plasm (Knaut et al., 2000; Yoon et al., 1997). The localization of RNA molecules to the germ plasm depends on *cis*-acting RNA elements that reside within the 3′ untranslated region (UTR) as shown for the RNA of *vasa* (Knaut et al., 2002). In addition to the enrichment of specific RNA molecules in the germ plasm, these RNAs are differentially stabilized and translated in PGCs and somatic cells (Köprunner et al., 2001; Wolke et al., 2002). Together, these post-transcriptional mechanisms, originally identified in *C. elegans* and *Drosophila* (Gavis and Lehmann, 1994; Seydoux and Fire, 1994; Seydoux, 1996), lead to the establishment of a distinct repertoire of proteins expressed in germ cells.

A thorough analysis of PGC behaviour and migration in zebrafish is facilitated by the extra-uterine development of the translucent embryo as well as by the ability to label PGCs with fluorescent proteins. These properties provide a unique opportunity to study cell migration and development at a high resolution within the intact organism. PGC migration in zebrafish is guided by binding of the chemokine stromal-cell-derived-factor (SDF)-1a, a small-secreted molecule that is dynamically expressed in the regions toward which the PGCs migrate (Doitsidou et al., 2002), to its receptor, CXCR4b (Doitsidou et al., 2002; Knaut et al., 2003). Whereas the development and migration of zebrafish PGCs have been studied in great detail starting at mid-gastrulation stages when the cells exhibit active guided migration [7-8 hours post fertilization (hpf)] (Braat et al., 1999; Knaut et al., 2002; Reichman-Fried et al., 2004; Weidinger et al., 2003; Weidinger et al., 1999; Weidinger et al., 2002; Yoon et al., 1997), earlier stages have not yet been examined. PGC migration in mouse has been studied in vivo a day after the onset of gastrulation (E7.5) a time point when the PGCs already exhibit migratory properties (Anderson et al., 2000).

Here we describe the cloning of the regulatory upstream region of the *askopos* gene (encoding a novel nuclear protein whose RNA is expressed in the germ plasm and the PGCs) and fusing it to a reporter gene and to RNA elements directing stabilization and translation to the germ cells. Generating transgenic fish for this construct allowed us to monitor PGC

development in live zebrafish embryos starting from the earliest stages of their development. This analysis revealed three phases in early PGC development. We found that immediately after their specification (about 3 hpf), PGCs have simple cell morphology. This stage is followed by a phase at which the cells assume complex cell morphology but do not change their position and do not respond to SDF-1a. During the third phase, a transition into a 'migratory stage' occurs as PGCs become responsive to directional cues provided by somatic cells secreting the chemokine SDF-1a. Furthermore, the transition to this stage is accompanied by a reduction in E-cadherin levels and depends on the function of the RNA-binding protein Dead end as well as on de novo transcription in the zygote.

Materials and Methods

Zebrafish strain and fish maintenance

Zebrafish (*Danio rerio*) of the AB genetic background were maintained, raised and staged as previously described (Westerfield, 1995; Kimmel et al., 1995).

Identification of the *askopos* (*kop*) gene

The *kop* cDNA (GenBank accession no. AY572416) was identified in a large-scale screen for genes expressed in a tissue-specific manner during early zebrafish development (Thisse et al., 2001). 3′- and 5′-RACE analysis confirmed that the original cDNA clone represents the full-length mRNA.

Alteration of Kop activity by RNA injections

Capped sense RNAs were synthesized using the Message Machine Kit (Ambion) and injected according to standard procedures. The pSP64-*kop-globin*-3′UTR construct contains the *kop* open reading frame (ORF) cDNA cloned into the pSP64TS vector, which includes the untranslated regions of the Xenopus *globin* gene. Injected RNA synthesized from this construct is inherited and translated by all cells of the embryo. The pSP64-*kop-nos*1-3′UTR construct contains the *kop* open reading frame cloned upstream of the *nanos*1 3′UTR. Injected RNA produced from this construct is translated and stabilized specifically in the PGCs.

Site-directed mutagenesis was performed by PCR using the original *kop* clone (cg 1111) as a template to obtain the pSP64-DN*kop-globin*-3′UTR construct. Two nucleotide mismatches were introduced leading to the exchange of Gly280 with Ser280 in the ATP/GTP-binding site motif A (P-loop; ANLTSGKT) of Kop. Injected RNA synthesized from this construct is expressed in a similar pattern to that of pSP64-*kop-globin*-3′UTR RNA (data not shown).

Kop subcellular localization and *kop* morpholino activity

The pSP64-*kop-gfp–nos*1-3′UTR construct includes the fusion of *kop* and *gfp* ORFs upstream of the 3′UTR of *nanos*1. The *kop* morpholino (5′-GAATGGATGTATCTGTGAATGACAT-3′) and the standard control morpholino were produced by GeneTools, OR. For the knockdown experiments: 0.6-5.4 pmol of these morpholino antisense oligonucleotides dissolved in 10 mM HEPES (pH 7.6) were injected into 1-cell-stage wild-type embryos. To examine the specific function of the *kop* morpholino, *DsRedex-nos*1-3′UTR and *kop-gfp–nos*1-3′UTR (each 300 pg RNA) was co-injected with 0.6 pmol of *kop* morpholino.

kop promoter cloning

RZPD Zebrafish genomic PAC-library 706 was screened for *kop* clones. Positive clones were identified and PAC clone BUSMP706O19135Q2 was used to clone a 4.9 kb region upstream of the first exon of *kop*.

Cloning of kop-EGFP-F-nos1-3′UTR construct and generation of transgenic fish

The kop-EGFP-F-nos1-3′UTR promoter construct was generated by PCR amplification of the kop promoter region using the forward primer (GAGCTCGCCACTGCATCATCCATTC) and the reverse primer (GACTAGTCCGAATGATATTTTTGTAG). The promoter region was cloned upstream of EGFP containing the farnesylation sequence (EGFP-F) and the 3′UTR of *nanos*1. This construct was introduced into the I-*Sce*I-pBSII-SK+ vector and the purified plasmid DNA was injected into 1-cell-stage fish embryos as previously described (Thermes et al., 2002). A female fish carrying the transgene was identified by screening for GFP-expressing PGCs among the progeny of injected females.

Constructs for RNA expression, knockdown and drug experiments

The pSP64-*gfp-nos*1-3′UTR (210 pg) and the pSP64-*E-cadherin*1-*nos*1-3′UTR (90 pg) constructs contain the *gfp* or the full-length zebrafish *E-cadherin*1 open reading frame (ORF) respectively followed by the 3′ untranslated regions of the *nanos*1 gene. The RNAs produced from these constructs are translated and stabilized preferentially in the PGCs. Dead end morpholinos (*dnd*-MO, 1200 pg) were injected as described in (Weidinger et al., 2003). The transcription inhibitor α-amanitin (Sigma) was injected into 1-cell-stage embryos as previously described (Kane et al., 1996).

In situ hybridization

One-colour whole-mount in situ hybridization was performed as described previously (Jowett and Lettice, 1994) with modifications described elsewhere (Hauptmann and Gerster, 1994; Weidinger et al., 2002).

Fluorescence microscopy and imaging of live cells

Images were obtained using a Zeiss Axioplan2 microscope controlled by the Metamorph software (Universal Imaging). Low magnification time-lapse movies were generated using a 10× objective at a rate of one frame per minute. High magnification time-lapse movies were generated using a 63× or 40× objective capturing frames at 10-second intervals. For time-lapse analysis, *kop*-EGFP-F-*nos*1-3′UTR transgenic females were crossed to wild-type males. For the E-cadherin forced expression experiments, *gfp-nos*1-3′UTR RNA was injected into 1-cell-stage wild-type embryos to label PGCs. Speed measurements of migrating PGCs were performed using the Metamorph software (Universal Imaging).

Cell transplantation

Attraction assay

Wild-type 1-cell-stage donor embryos were injected with 240 pg *sdf-1a-globin*-3′UTR RNA [morpholino-resistant *sdf-1a*; (Doitsidou et al., 2002)], *sdf-1a* and *sdf-1b* morpholino (0.4 pmol each) and dextran Alexa Fluor 568 (10,000 kDa; Molecular Probes, 0.15 ng). Progeny of kop-EGFP-F-*nos*1-3′UTR transgenic females injected at the 1-cell stage with *sdf-1a* and *sdf-1b* morpholino (0.4 pmol each) served as hosts. In transplantation experiments, cells from 5.3 hpf donor embryos were transferred into 3 hpf and 5.3 hpf host embryos.

Heterochronic PGC transplantations

One-cell-stage embryos were injected with *DsRedex*1-*globin*-3′UTR (300 pg) and *gfp-nos*1-3′UTR (240 pg) RNA served as donors from which PGCs were isolated at 12 hpf. mGFP labelled PGCs were transplanted into 3 hpf wild-type or α-amanitin injected host embryos. Lack of cells expressing only DsRed served as an indication that no somatic cells were co-transplanted with the PGCs. Conversely, progeny of *kop*-EGFP-F-*nos*1-3′UTR transgenic females injected with α-amanitin at the 1-cell-stage served as donors from which PGCs were isolated at 7 hpf and transplanted into 5.3 hpf *egfp*-f-*globin*-3′UTR (6 pg, to observe membranes of somatic cells) injected wild-type host embryos.

Immunohistochemistry

Embryos were fixed with 4% paraformaldehyde/PBS for 2 hours at room temperature. After fixation, the embryos were washed three times for 5 minutes with PBTX (PBT, 0.2% Triton X-100) and subsequently blocked with PBTB (PBT, 0.2% Triton X-100, 1% BSA) for 1 hour. The embryos were incubated in the blocking solution containing the primary antibody overnight at 4°C. GFP antibody was obtained from Santa Cruz Biotechnology and anti human E-cadherin antibody from Transduction Laboratories. The embryos were then washed with PBTX 8 times for at least 30 minutes each. Thereafter, they were incubated with the secondary antibody (1:200 Cy2-conjugated anti-rabbit polyclonal, Jackson ImmunoResearch Laboratories; 1:200 Alexa Fluor546-conjugated anti-mouse IgG, Molecular Probes) overnight at 4°C. Finally, embryos were washed with PBTX for several hours and observed by confocal microscopy (Leica TCS SL).

In vivo staining of activated caspases

Embryos were grown at 28°C in 0.3× Danieau's solution. After removal of the chorion, the embryos were incubated in 300 μL of 0.3× Danieau's solution plus 1 μL of Red-VAD-FMK (sulf-rhodamine conjugated VAD-FMK caspase family inhibitors, BIOCAT) for 1 hour at 30°C. The embryos were then washed with Wash Buffer (BIOCAT) for at least 5 minutes under slow agitation. The embryos were then mounted and observed under a Zeiss Axioplan2 fluorescence microscope controlled by Metamorph software (Universal Imaging). Caspase positive cells show bright red signal, whereas negative cells show weak background signal. The *diphtheria toxin A-chain* open reading frame (ORF) cDNA was cloned into the pSP64 vector. RNA synthesized from this construct was co-injected with Oregon Green 488 (Dextran 70,000 kDa, Molecular Probes) into one of 64 blastomeres (2 hpf) of wild-type embryos (0.15 pg *diphtheria toxin* RNA, 6.3 ng Oregon Green 488). The transcription inhibitor α-amanitin (Sigma) was injected into 1-cell-stage embryos as previously described (Kane et al., 1996). Dead end morpholinos (*dnd*-MO, 1200 pg) were injected as described previously (Weidinger et al., 2003). For the α-amanitin or *dnd*-MO injection, *kop*-EGFP-F-*nos*1-3′UTR transgenic females were crossed to wild-type males to obtain embryos whose PGCs were labelled with GFP.

Confocal-data analysis

Green channel (PGC membrane) and red channel (E-cadherin) pictures, recorded on a Leica TCS SL confocal microscope were analysed by using the Metamorph software (Universal Imaging) (see Fig. 4B).

Results

The askopos gene is expressed in the zebrafish germline and encodes a nuclear protein

The *askopos* (*kop*) gene (*askopos*, 'without purpose' in Greek) was identified in a screen for genes that are expressed in zebrafish PGCs (Thisse et al., 2001). Maternally provided *kop* RNA is uniformly distributed at the 1-cell-stage embryo and becomes enriched at the cleavage furrows at 0.75 hpf and 1 hpf (2- and 4-cell stages) (supplementary material Fig. S1A and data not shown). The RNA is then expressed in the PGCs as they migrate towards the gonad and can no longer be detected in 5-day-old embryos (supplementary material Fig. S1A and data not shown). This expression pattern is comparable to that of *nanos*1 and *dead end* that play a role in early PGC development in zebrafish, and differs from that of *vasa* that is continuously expressed in the germline (Köprunner et al., 2001; Weidinger et al., 2003; Yoon et al., 1997). *kop* mRNA encodes a novel 335 amino acid protein which contains an ATP/GTP-binding site motif A referred to as P-loop (Saraste et al., 1990) (supplementary material Fig. S1B). Interestingly, no homologues to the Kop protein could be identified in other species and hence the molecular function of such proteins is unknown. Therefore, we first wished to determine the subcellular localization of the Kop protein. A Kop-GFP fusion protein expressed in the PGCs revealed its localization to the nuclei (supplementary material Fig. S1C), suggesting that its function could be exerted in the nucleus.

Alterations in the level of Kop activity

We initially overexpressed *kop* RNA throughout the embryo by injecting RNA containing *kop* open reading frame fused to globin 3′UTR. Despite the high amounts of injected RNA (up to 900 pg of *kop-globin*-3′UTR RNA per embryo) we could not detect abnormalities in either somatic or PGC development. Similarly, overexpression of *kop* in the PGCs by injection of *kop-nos*1-3′UTR RNA had no effect on PGC development, which arrived at the region of the gonad and expressed *vasa* RNA (data not shown).

Subsequently, we aimed at reducing Kop's activity using modified antisense oligonucleotides (morpholinos, *kop*-MO). Injection of antisense oligonucleotides directed against the 5′ of *kop* RNA effectively blocked the translation of the *kop-gfp-nos*1-3′UTR RNA (supplementary material Fig. S1C). Nevertheless, we failed to detect any abnormalities in PGC development as judged by normal migration of the cells and normal expression of *vasa* RNA (data not shown).

As increasing the amount of *kop* RNA as well as inhibiting its translation had no effect on PGC development, we reasoned that similar to other examples in zebrafish (e.g. Gritsman et al., 1999) maternally provided Kop protein could be sufficient to carry out the early function of the gene. We therefore attempted to inhibit the function of such putative maternal protein by introducing a dominant negative (DN) form of Kop. A mutated form of the Kop protein was constructed by exchanging a conserved glycine in the P-loop motif with a serine residue. However, overexpression of this protein by injecting embryos with DN*kop-globin*-3′UTR RNA did not affect PGC development.

As none of the manipulations appear to affect normal PGC development it is possible that the function of Kop is redundant, that our knockdown approaches did not reduce its activity to a level low enough to be phenotypically manifested

or as suggested by its name, the protein does not play an essential role in PGC development.

Employing the *kop* promoter to establish a transgenic fish line expressing EGFP-F during early PGC development

To follow PGC migration in live embryos, we have routinely labelled these cells with GFP relying on 3′UTR elements that confer specific expression in PGCs (Köprunner et al., 2001; Wolke et al., 2002). Others have used transgenic fish in which GFP was flanked by an upstream EF1α or *vasa* promoter and by the downstream 3′UTR of the *vasa* gene (Knaut et al., 2002; Krøvel and Olsen, 2002). Monitoring PGCs in live embryos using either one of these approaches allowed clear visualization of the cells albeit not earlier than mid-gastrulation stages (ca. 7 hours of development).

The strong expression of *kop* in early embryos prior to the initiation of zygotic transcription (see supplementary material Fig. S1) implies that its RNA is transcribed during oogenesis. We have taken advantage of this finding and sought to monitor the behaviour of the PGCs from the earliest stages of their development by generating a construct in which the *kop* promoter was positioned upstream to EGFP containing a farnesylation signal (EGFP-F) followed by the 3′UTR of the *nano*s1 gene (Fig. 1A). This construct was injected into 1-cell-stage embryos and a transgenic line carrying the construct was established. Heterozygous females carrying one copy of the transgene were mated with wild-type males and the progeny (half of which inherited the transgene) was analysed by in situ hybridization using EGFP as a probe. Both transgenic and non-transgenic offspring exhibited the same RNA expression pattern as shown in Fig. 1B, consistent with the idea that the *kop* promoter is active prior to the first meiotic division. The RNA of the transgene is ubiquitously distributed at 1-cell stage (data not shown) and becomes enriched in the cleavage planes at 0.75 hours post fertilization (hpf) and 1 hpf (2- and 4-cell stages) (Fig. 1B). This result indicates that similar to the 3′UTR of the *vasa* gene (Knaut et al., 2002; Krovel and Olsen, 2002), the 3′UTR of *nanos*1 is sufficient for localizing the RNA to the germ plasm. Importantly, the localization of the *nanos*1 3′UTR-containing RNA to the region of the germ plasm (Fig. 1B) is only rarely observed when a similar in vitro transcribed RNA is injected after fertilization (data not shown). It is therefore likely that during oogenesis the 3′UTR of *nanos*1 becomes associated with other maternal components that facilitate its efficient localization to the germ plasm. Later, expression of *egfp-f* RNA is observed in PGCs during their migration toward the region of the gonad and can be detected in these cells during the next three days (Fig. 1B and data not shown). In agreement with the notion that the *kop* promoter is strictly maternal, the progeny of transgenic males mated with wild-type females showed no expression of *egfp-f* RNA.

Early EGFP expression in the PGCs

In progeny of transgenic females EGFP is detectable in PGCs starting at 3 hpf (Fig. 1C). Only faint uniform EGFP protein expression could be detected prior to PGC specification (2.75 hpf in Fig. 1C) unlike the strong somatic expression of GFP

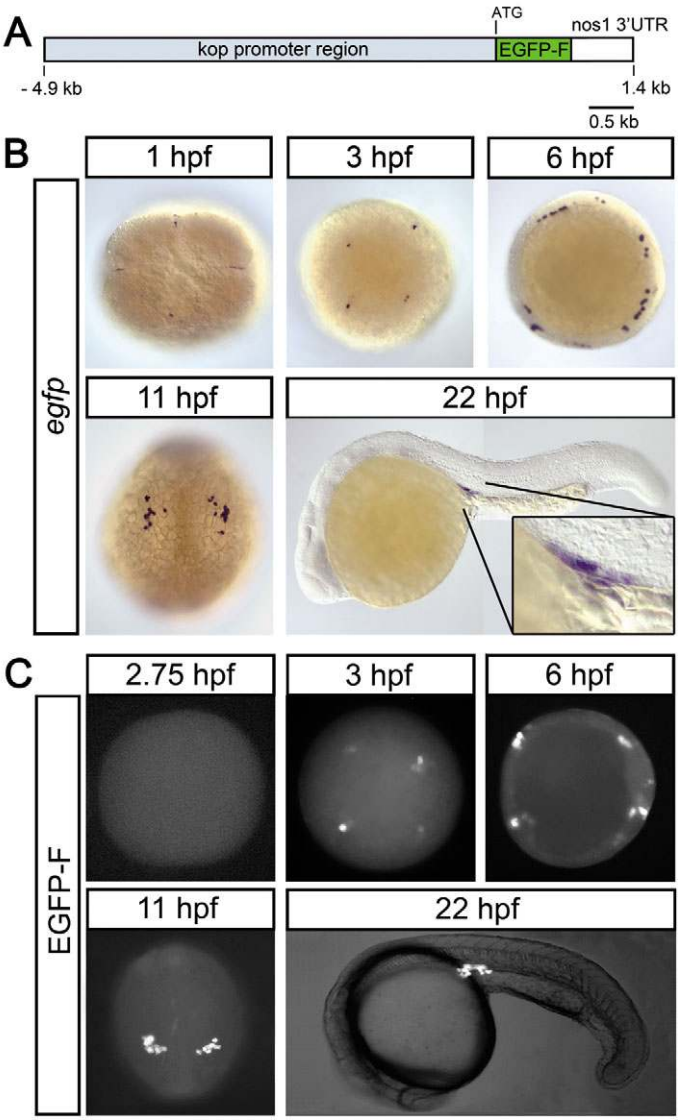

Fig. 1. Early labelling of PGCs in live embryos. (A) Schematic representation of the *kop* promoter region fused to EGFP-F-*nos*1-3′UTR that was used to generate transgenic fish. (B) Whole mount in situ hybridization of progeny of transgenic females showing the distribution of *egfp* RNA. The inset included in the image depicting a 22 hpf embryo shows a magnification of the region where the PGCs normally reside. (C) Specific expression of EGFP-F in the zebrafish germline starting at the earliest stages following its formation. No specific EGFP-F expression prior PGC specification (e.g. 2.75 hpf) can be detected.

that is observed when other promoters and *vasa*-derived RNA elements are used (Knaut et al., 2002; Krovel and Olsen, 2002). Thus, owing to the *kop*-EGFP-F-*nos*1-3′UTR transgene, PGCs can be visualized with great clarity in live embryos as soon as they are specified. Furthermore, PGCs could be isolated by fluorescent activated cell sorter (FACS) at different stages and in conjunction with the *cis*-acting elements described above any gene of interest could be specifically expressed early in the germline. Lastly, this line should significantly improve a recently described method for germline replacement by allowing more efficient transplantation of mutant germ cells

into wild-type hosts (Ciruna et al., 2002). In the context of this study, the *kop*-EGFP-F-*nos*1-3′UTR transgenic line enabled us to examine PGCs in live embryos during stages that have not been previously amenable to investigation.

The onset of PGC migration in zebrafish

When PGC migration was originally described based on the analysis of fixed embryos, the first stage of active migration was shown to occur at about 5.5 hpf (before shield stage) (Weidinger et al., 1999). This type of analysis provided no information regarding the dynamic cellular mechanisms responsible for promoting the transition of the cells into a motile phase and the acquisition of responsiveness to guidance cues. Such a transition in cell behaviour is exhibited by many cell types in normal development (e.g. Christiansen et al., 2000; Savagner, 2001) and disease (e.g. Thiery, 2002). For example, following their specification, neural crest cells delaminate from the neuroepithelium of the developing neural tube and become migratory (Halloran and Berndt, 2003; Savagner, 2001). A comparable transition is observed in border cells that delaminate from an epithelium and invade a different cellular environment on their way to the target tissue (Rørth, 2002; Starz-Gaiano and Montell, 2004). Similarly, cancer cells undergo changes in the cellular phenotype as they leave an organized epithelial layer, acquire an invasive phenotype and initiate metastasis (Thiery, 2002). To characterize the behaviour of the PGCs during this critical time point, we monitored the cells from 3 hpf using low magnification time-lapse analysis. At this stage, PGCs are found in four clusters and show no movement relative to their somatic neighbours (Fig. 2A). It is only at about 4.5 hpf (the end of dome stage), about 1.5 hours after PGCs exhibit specific *nos*1-3′UTR controlled EGFP-F expression, that a few cells start leaving the clusters (Fig. 2A) (supplementary material Movie 1).

To study these early steps of PGC behaviour at a higher resolution, we observed the cells at these stages (3 to 4.7 hpf)

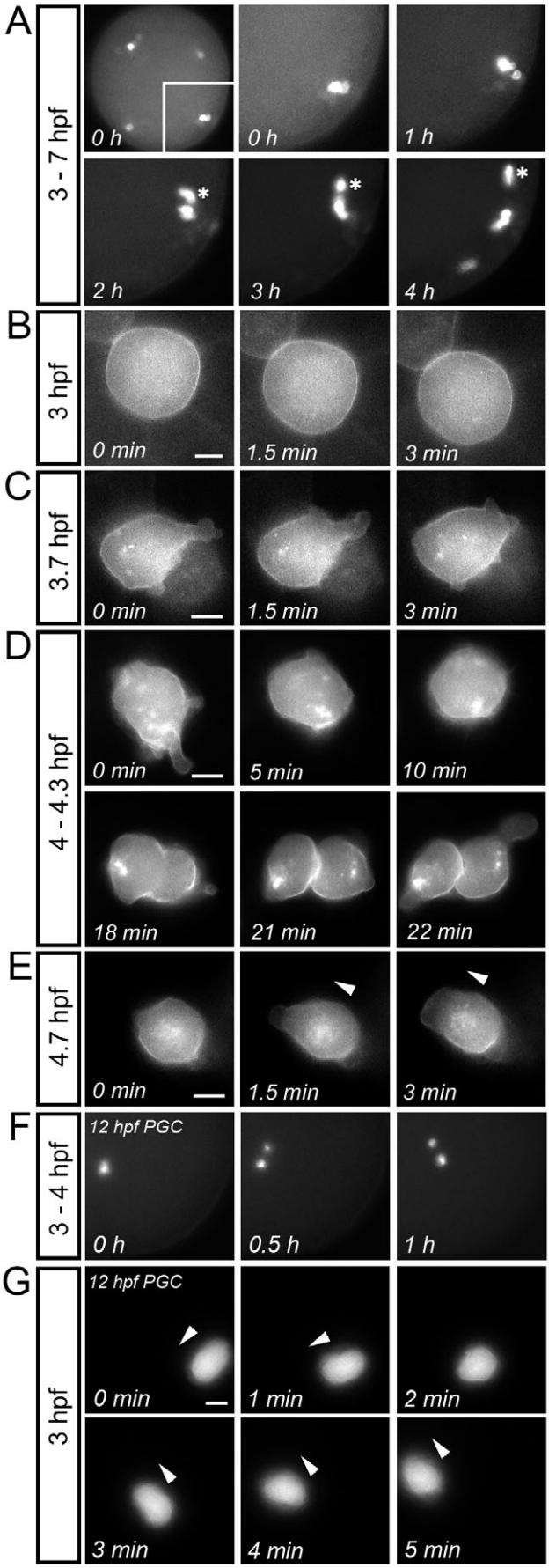

Fig. 2. Marked alterations in migratory behaviour are detectable during earliest stages of PGC development. (A) Snapshots from a low magnification time-lapse movie recorded over 4 hours of zebrafish development. The area marked in the top left panel is magnified in the rest of the panels. The germ cells remain in their positions for about 1.5 hours following PGC specification and start to migrate actively around dome stage (4.5 hpf, supplementary material Movie 1, *n*=4 embryos). An asterisk marks a PGC, which leaves the cell cluster. (B-E) High-magnification snapshots from time-lapse movies of wild-type embryos (see supplementary material Movies 2-5). (*B*) At 3 hpf, PGCs exhibit simple morphology. (C,D) At 3.7 hpf (C) up to 4.3 hpf (D) the cells develop small, as well as long complex protrusions extended in random directions, yet are not polarized and do not migrate. At these stages, the cells show round morphology during cell divisions (D). (E) Soon after 4.3 hpf, PGCs exhibit polarized elongated cell morphology and extend pseudopodia in the direction of migration. (F) Snapshots from a low magnification 1-hour long time-lapse movie of PGCs from a 12 hours donor embryo in a 3 hpf host embryo. The transplanted cells migrate in the early host (supplementary material Movie 6, three cells in two embryos examined). (G) Transplanted cells similar to those in F show polarized elongated cell morphology and migration behaviour similar to PGCs after 4.3 hpf (supplementary material Movie 7, 40× magnification). The white scale bars represent 10 μm and the white arrowheads indicate the direction of migration.

by generating high magnification time-lapse movies. Based on cell morphology and motility we could define 3 phases of early PGC development. The first phase is characterized by simple PGC morphology and lack of migration (Fig. 2B) (supplementary material Movie 2, *n*=8/8 cells in 7 embryos). During the second phase that starts about 30 minutes later (3.3 hpf), the germ cells extended primarily multiple small protrusions in all directions as well as unusually elongated protrusions (*n*=14/14 cells in 11 embryos), but still showed no net movement (Fig. 2C) (supplementary material Movie 3). This complex morphology that is observed during the next 40 minutes disappears when the cells undergo mitosis (Fig. 2D) (supplementary material Movie 4). In the last phase, which starts at about 4.5 hpf, a few polarized cells extending broad pseudopodia characteristic of migratory PGCs (Reichman-Fried et al., 2004) show bona-fide active migration. The proportion of such cells gradually increased so that at 4.7 hpf about 30% of cells assumed migratory behaviour and about 50% of PGC showed polarized cell morphology (Fig. 2E) (supplementary material Movie 5; 8/11 cells in 8 different embryos). At 5.5 hpf about 75% of cells show active migration and at 6 hpf this number increased to more than 90% of all PGCs.

Whereas the phases described above are likely to represent steps in the cellular maturation of PGCs, it is formally possible that they actually reflect alterations in the properties of their early environment. To test this possibility, we have transplanted migratory PGCs into early host embryos (namely, 12 hpf PGCs into 3 hpf embryos). Notably, the transplanted cells exhibited cellular behaviour characteristic of their developmental stage when present in early host embryos whose endogenous PGCs show no movement (Fig. 2F,G) (supplementary material Movies 6, 7; *n*=4/5 cells in 3 embryos). Indeed, the transplanted cells reached the site where the gonad develops by the end of the first day of development (data not shown). The transplanted PGCs exhibited polarized formation of protrusions and importantly, occasionally exhibited 'run and tumble' behaviour (Reichman-Fried et al., 2004). Namely, we occasionally observed loss of cell polarity coupled with a change in the direction of migration. This experiment thus clearly demonstrated that early in development, PGC competence to polarize and migrate constitutes an autonomous property of these cells.

de novo transcription in the zygote and dead end function are essential for the cellular maturation of PGC

To determine whether zygotically transcribed genes are involved in the cellular maturation of zebrafish PGCs we have treated the embryos with the RNA polymerase II and III inhibitor α-amanitin (Jacob et al., 1970). As previously described (Kane et al., 1996), inhibition of zygotic transcription (assayed by the effect on *notail* transcription) (Fig. 3B) did not affect cell division, but had a dramatic effect on early gastrulation movements, most notably on epiboly (Fig. 3A). Despite the strong effect of this treatment on morphgenetic movements, we could not observe any indications for an effect on PGC survival. Specifically, we could not detect activation of caspases that would indicate the initiation of caspase dependent programmed cell death in the PGCs (supplementary material Fig. S2B; 12/12 embryos) and could not observe dying PGCs for as long as we followed the treated cells (until 9 hpf, that is 4.5 hours after they normally become motile, supplementary material Fig. S2B and Fig. S2C). Interestingly, an adverse effect on PGC migration was observed in α-amanitin treated embryos as evident by PGCs remaining in their original cluster several hours following the time at which they normally become motile (Fig. 3C) (4/4 embryos). Strikingly, the inhibition of transcription brought PGC maturation to a halt as manifested by lack of motility and polarity while extending protrusions in all directions. These characteristics signify an arrest of PGC differentiation at the second phase (Fig. 3D) (supplementary material Movies 8, 9; 11/11 cells). As the α-amanitin treatment affected both the transcription in the PGCs as well as in somatic cells, we sought to determine the relative contribution of each one of these populations to the observed phenotype. To this end, we have transplanted motile PGCs from untreated embryos into α-amanitin treated hosts and examined their behaviour. Interestingly, whereas we found that the transplanted cells exhibited morphological behaviour characteristic of their developmental stage as they were producing broad pseudopodia typical of migrating PGCs (Fig. 3E) (11/11 cells), they did not migrate relative to the α-amanitin treated somatic cells. As we ruled out the possibility that α-amanitin from neighbouring cells can affect transcription in cells that did not receive the chemical themselves (data not shown), the result of this experiment points to the requirement for active transcription in surrounding somatic cells, presumably for providing PGCs with the extracellular matrix or cell adhesion conditions that are permissive for active migration. Conversely, α-amanitin treated PGCs transplanted into untreated host

Fig. 3. Zygotically transcribed genes are essential for PGC polarization and motility. (A) Embryos treated with α-amanitin (lower panels) exhibit gastrulation defects, most pronounced is the inhibition of epiboly (arrowheads). (B) Transcription of the zygotically expressed genes [represented here by RT-PCR analysis for *no tail* (*ntl*)] is inhibited by α-amanitin (lower panel) whereas the level of maternally provided transcripts [represented here by RT-PCR analysis for *vasa* (*vas*)] is unaffected. (C) Snapshots from low-magnification time-lapse movies recorded over 2 hours of zebrafish development showing a representative PGC cluster. The germ cells in control embryos (upper panels, one cell labelled with an asterisk) migrate actively and leave the cluster following the transition whereas PGCs in α-amanitin treated embryos (lower panels) remain clustered and immotile. (D) Snapshots from high-magnification time-lapse movies of control (upper panels) and α-amanitin treated PGCs (lower panels). PGCs treated with α-amanitin remain arrested in the second phase of their differentiation extending protrusions in all directions and fail to polarize (supplementary material Movies 8 and 9). (E) Snapshots from high-magnification time-lapse movies. 12 hpf old wild-type PGCs transplanted into an α-amanitin treated host embryo are not able to migrate relative to host cells, but show cell morphology characteristic of cells of their age. (F) 7 hpf old α-amanitin treated PGCs transplanted into a 5.3 hpf wild-type host remain arrested in the second phase of their maturation displaying extensions in all directions and fail to polarize and migrate. The apparent movement of the cluster in the lower panels is a result of passive movement together with somatic cells. (G) At 4.3 hpf, Dead end knockdown (*dnd*-MO) PGCs behave like wild-type cells of a similar age. At 4.7 hpf, *dnd*-MO treated germ cells do not polarize and migrate but are capable of extending small protrusions and divide (supplementary material Movies 10 and 11). Bars, 10 μm.

embryos displayed morphological characteristics and protrusive activity suggesting that they are arrested in the second phase of PGC maturation. These cells exhibit multiple small as well as unusually elongated protrusions in all directions and did not migrate relative to the wild-type somatic cells (Fig. 3F, 6/8 cells). Importantly, the α-amanitin treatment did not affect PGC survival during the stages we analysed so that live GFP labelled PGCs could be observed in ectopic positions in 24 hours old embryos (data not shown). Together, these data suggest that maternal contribution of RNA and proteins is not sufficient for the transition of the PGCs to become polarized and perform active migration and that de novo transcription within these cells is essential for the process.

A gene product whose function is required for PGC motility in zebrafish is Dead end (Weidinger et al., 2003). To determine whether early steps in PGC development require the function of Dead end, we monitored the cells in embryos injected with *dead-end* morpholino antisense oligonucleotides (*dnd*-MO).

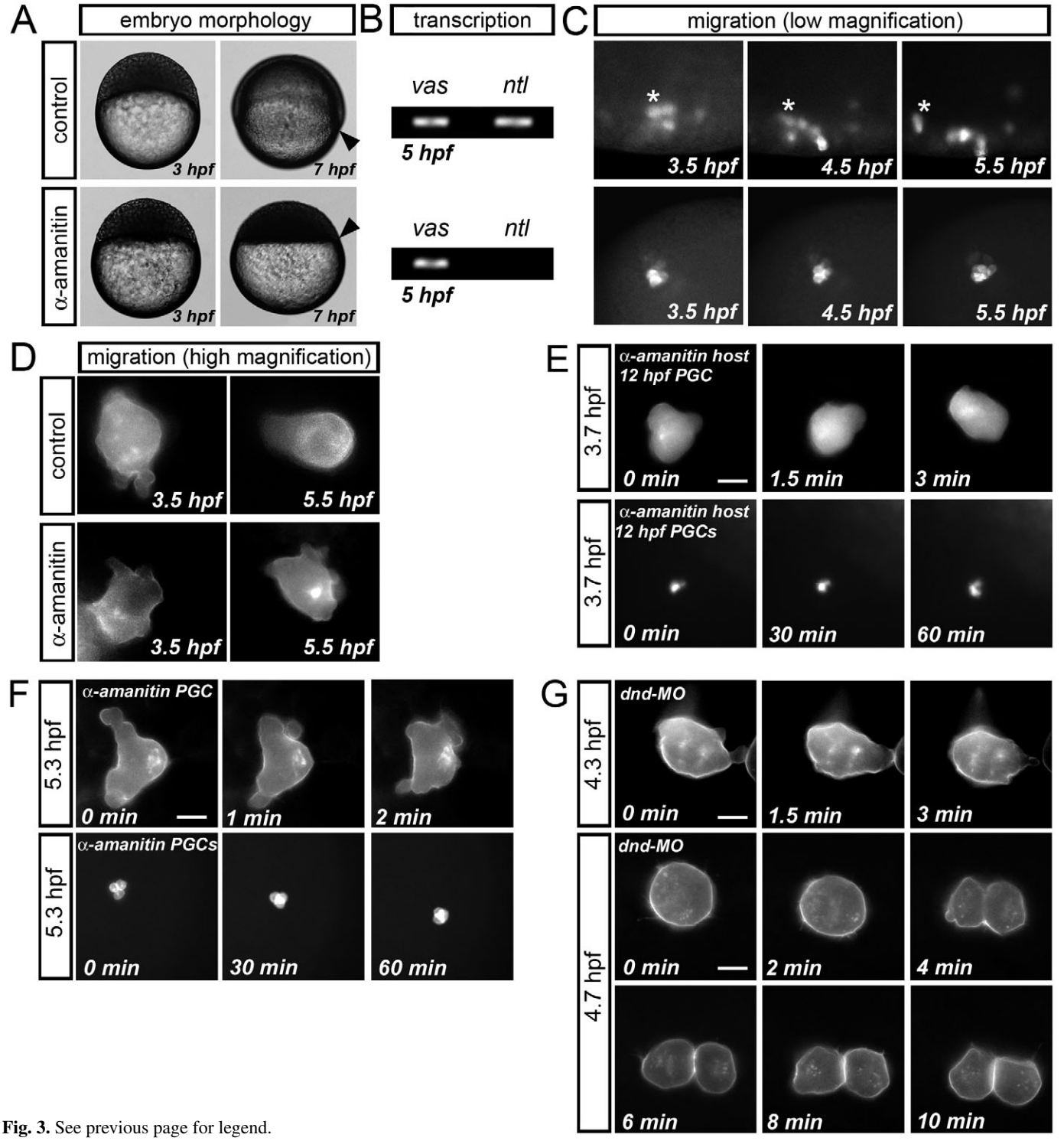

Fig. 3. See previous page for legend.

Whereas PGCs treated with *dnd-MO* eventually die (Weidinger et al., 2003), we could not detect activation of caspases that would indicate the initiation of caspase-dependent programmed cell death at the relevant stages (supplementary material Fig. S2B; 6/6 embryos). It is noteworthy to mention that PGC death was not observed before 14 hpf (supplementary material Fig. S2C), which is more than 8 hours after non-treated cells became motile and migrated towards the SDF-1a source. Interestingly, PGCs depleted for Dead end showed normal cell morphology and behaviour during the first two phases of their development (Fig. 3G) (and data not shown; see also supplementary material Movie 10; 14/14 cells in 11 embryos). Noticeably however, in embryos lacking Dead end the PGCs did not undergo the transition to the last phase at which they normally polarize, extend broad pseudopodia and actively migrate. PGCs in *dnd*-MO treated embryos nevertheless do exit the second phase of cellular development as they cease to extend the protrusions characteristic of this step (Fig. 3G) (supplementary material Movie 11; 9/10 cells in 8 embryos), unlike α-amanitin treated PGCs that appear to be arrested in this phase. Once Dead end knockdown PGCs exit the second phase of maturation, they show primarily simple cell morphology no polarization and do not migrate. Despite the fact that these cells eventually die, we believe the phenotype we observe reflects a specific requirement for Dead end function, as the treated cells are capable of extending filapodia as well as small protrusions and continue to divide (e.g. Fig. 3G) (supplementary material Movie 11). Dead end function is therefore dispensable for early maturation steps of PGCs but essential for attaining migratory properties.

The level of membranal E-cadherin is modulated during early PGC development

The segregation of individual cells from the tissues where they originally reside requires alterations in their adhesive properties. Modulations of cell-cell interaction leading to cell detachment and invasion of neighbouring tissues has been shown to promote dispersion of tumour cells and to be essential for morphogenesis during normal development (reviewed in Christofori, 2003; Tepass, 1999; Thiery, 2002; Thiery, 2003). A molecule known to play a critical role in controlling cell-cell adhesion in such biological contexts is the calcium-dependent cell adhesion molecule E-cadherin (Wheelock and Johnson, 2003).

To determine whether changes in E-cadherin distribution could account for the transition in cell behaviour described above, we have assessed the level of the protein on the PGC membrane at the relevant developmental stages. We have considered the possible variability in staining intensity of E-cadherin in PGCs derived from embryos of the same or different stages and normalized this value by presenting it relative to that of an adjacent somatic cell (Fig. 4B). Interestingly, at stages at which wild-type PGCs are migratory, a significantly lower level of membranal E-cadherin was observed relative to that detected at pre-migratory stages (Fig. 4A,C). Furthermore, the temporal correlation between E-cadherin expression and PGC motility could be extended to cases in which the PGCs were rendered immotile experimentally. As we have previously described, reducing the level of the Dead end protein in the PGCs results in a severe migration phenotype as a result of complete inhibition of cell motility. Consistent with the notion that a reduction in E-cadherin levels is important for PGC motility, we found that in immotile Dead end knocked-down PGCs E-cadherin is not down regulated (Fig. 4A,C). Whereas we expressed the alterations in E-cadherin levels as a ratio with the level observed in somatic cells, a similar result is observed when measuring the absolute signal level on the membrane of the PGCs (data not shown). Although E-cadherin is down regulated on the membrane of PGCs upon onset of migration, its expression persists in the cells as they migrate. This finding is consistent with the idea that similar to PGCs and other cell types in different organisms (Bendel-Stenzel et al., 1998; Dumstrei et al., 2002; Jenkins et al., 2003; Montero and Heisenberg, 2004; Niewiadomska et al., 1999), zebrafish germ cells probably require a certain level of E-cadherin for their proper migration and cell morphology. To test this notion more directly, we have forced expressed E-cadherin in the PGCs and monitored their behaviour. Indeed, PGCs forced expressing E-cadherin exhibited a dramatically reduced migration speed with some cells that remain on the spot while extending protrusions in all directions (Fig. 4D,E) (4/4 embryos). This cellular behaviour resulted in a large proportion of embryos with cells located in ectopic positions (80% compared with 8% in the control embryos having 4 or more ectopic PGCs). Overall, 30% of the PGCs were found in ectopic positions in 22 hpf experimental embryos versus less than 5% of control RNA injected embryos (Fig. 4F,G). This phenotype is milder than that observed when CXCR4b or SDF-1a are knocked down, consistent with the idea that E-cadherin level is important for optimal migration speed rather than cell guidance.

Acquistion of PGC responsiveness to SDF-1a represents a cellular differentiation step that is temporally distinct from their specification

The ability to observe PGC behaviour in vivo at the earliest stages of their development allows us to investigate the progression of the cellular response to external cues namely, to SDF-1a, the chemokine that directs PGC migration (Doitsidou et al., 2002). The lack of PGC migration at early stages could result from high levels of SDF-1a expression where they reside similar to the situation found at their intermediate or final targets (Reichman-Fried et al., 2004). Alternatively, this behaviour could reflect an intermediate stage during the cellular differentiation of PGCs at which the cells are not yet capable of responding to directional cues.

Using a combination of RT-PCR and in situ hybridization experiments we have determined that *sdf*-1a is transcribed only zygotically and that the initial uniform distribution of the transcripts is converted into a restricted pattern of expression at 4.7 hpf (data not shown). At this stage, *sdf*-1a is restricted to the blastoderm margin, the position at which the PGCs reside. As the RNA of the SDF-1a receptor, *cxcr4b*, is maternally provided (Chong et al., 2001) (data not shown), the PGCs could supposedly respond to SDF-1a upon specification. To determine whether local high concentration of SDF-1a expression is indeed responsible for the lack of cell movement, we inhibited its translation using morpholino antisense oligonucleotides. Significantly, this manipulation had no effect on the early behaviour of the PGCs (data not shown).

We have subsequently sought to determine the responsiveness of PGCs to SDF-1a at these early stages. To this end, we have transplanted cells overexpressing SDF-1a into embryos whose endogenous SDF-1a activity was inhibited and examined the response of the EGFP-F-labelled PGCs. PGCs exposed to the ectopic SDF-1a source at 3 hpf did not migrate toward the explant for at least 1.5 hours nor did they polarized in the direction of the attractant source (Fig. 5A) (supplementary material Movie 12; 4/4 embryos) suggesting that at this stage they lack some components required for responding to the signal. Notably, performing a similar transplantation experiment using older host embryos (older than 4.5 hpf) led to a rapid response (5 minutes after transplantation) of host PGCs that migrated toward the SDF-1a expressing cells (Fig. 5B) (supplementary material Movie 13, 9/9 embryos). In control experiments, where the transplanted cells did not express SDF-1a, the PGCs were motile and able to polarize but were not attracted to the explant (Fig. 5C) (supplementary material Movie 14; 2/2 embryos). Taken together, these results show that responsiveness to SDF-1a is acquired gradually at a late stage that is distinct from that at which PGCs are formed. Furthermore, despite extending protrusions, PGCs remain unresponsive to the chemoattractant and only 1.5 hours later can they initiate chemokine-guided

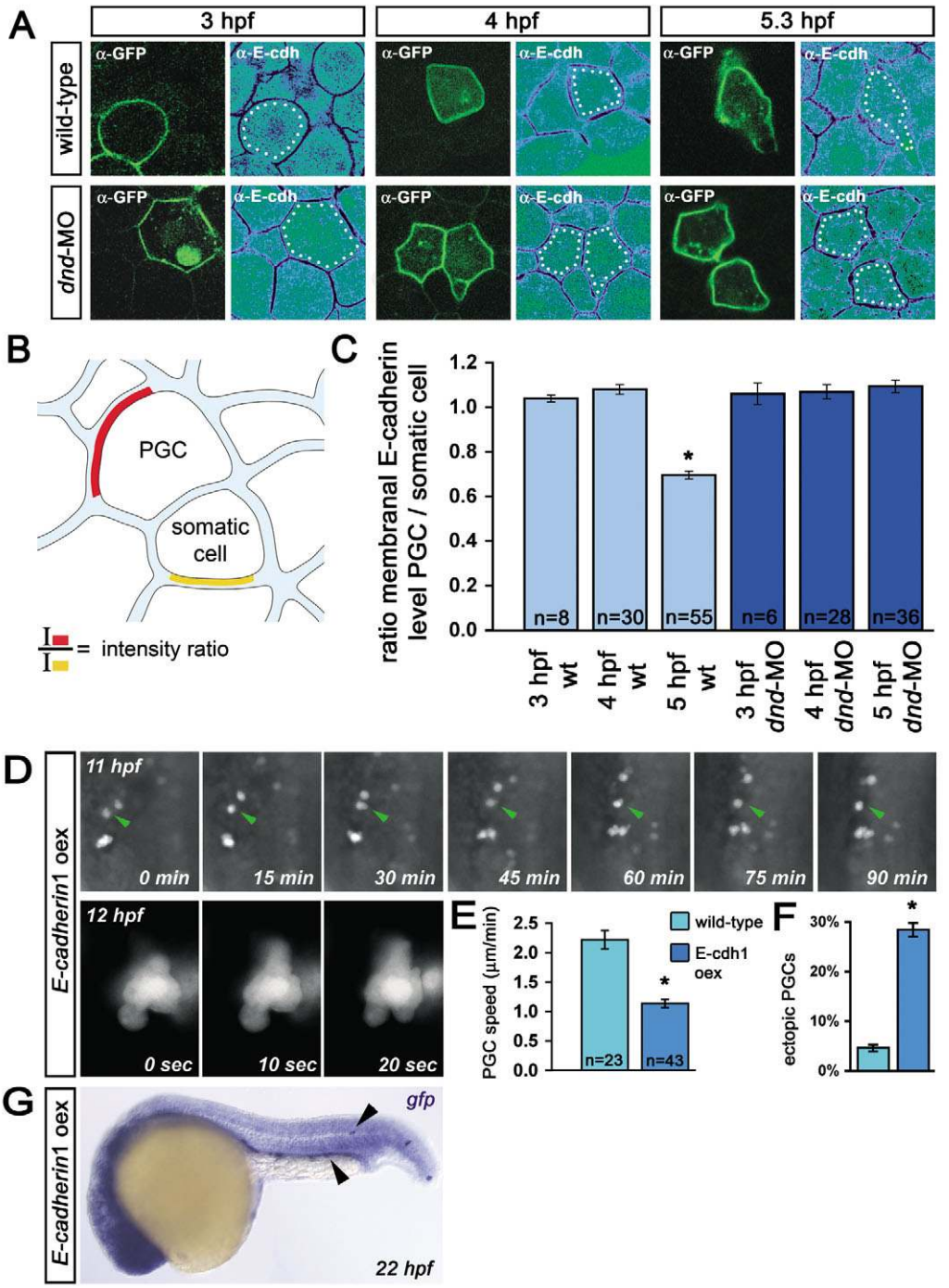

Fig. 4. E-cadherin is dynamically regulated during early stages of PGC development. (A) The expression level of E-cadherin protein is altered during early development in wild-type PGCs (top row, blue, the contours of the relevant cells are delineated with white dots), but remains constant in PGCs knocked down for Dead end (bottom row). (B) A graphic representation of the quantitative analysis examining the relative E-cadherin level on PGCs. Regions in PGC and somatic cell membranes were selected for analysis and the average pixel intensity obtained from the PGC membrane (the inner half of the membrane, red) was divided by the average pixel intensity of the somatic cell membrane (the inner half of the membrane, yellow). (C) Wild-type PGCs at 5.3 hpf show significantly reduced E-cadherin levels relative to earlier stages (comparing 5.3 hpf with 3 hpf). *dnd*-MO treated PGCs showed no such change in E-cadherin levels on the membrane. (D) PGCs in which full-length E-cadherin is forced expressed show a strong reduction in cell motility (e.g. the cell marked with the green arrow). The PGCs exhibit extensive non-polarized protrusive activity (high magnification snapshots). (E) PGC migration speed is severely reduced compared with wild-type PGCs. (F) Embryos forced expressing E-cadherin (122 embryos examined) in PGCs show 30% ectopic germ cells in comparison to 5% of control embryos (120 embryos examined). (G) In 22 hpf embryos in which E-cadherin is forced expressed in the PGCs, ectopic PGCs can be observed (black arrowheads, cells labelled using a GFP probe). In (C,E) n is the number of cells analysed, the error bars represent the standard error of the mean (s.e.m.), asterisk signifies $P<0.001$.

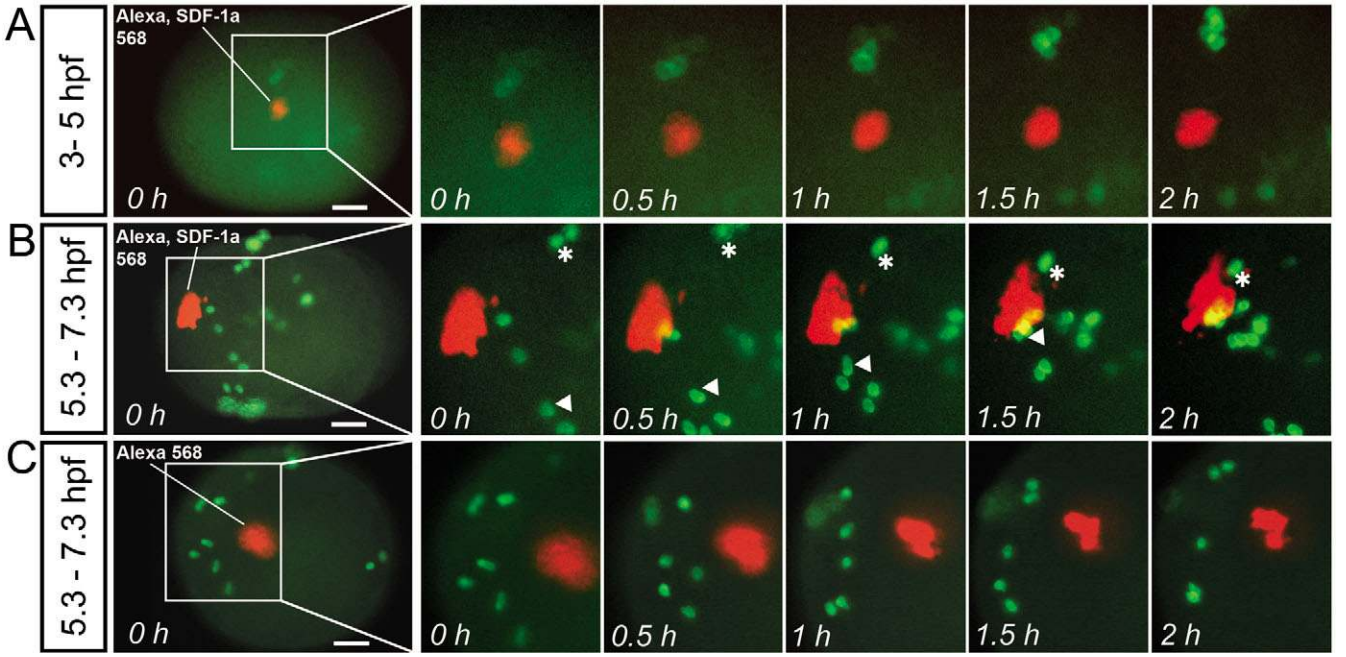

Fig. 5. The progression of PGC responsiveness to SDF-1a. (A) PGCs (green) do not respond to SDF-1a expressed by transplanted cells (red), from the time of their specification until 4.5 hpf. (B) At 5.5 hpf, the PGCs (green) migrate actively towards the SDF-1a expressing transplant (red) and remain in close proximity to it. Two PGCs migrating toward the SDF-1a source are marked with an asterisk and an arrowhead. (C) A control experiment shows that migrating PGCs (green) remain indifferent to transplanted cells (red) that do not express SDF-1a. The images were obtained from time-lapse movies generated for each experiment (see supplementary material Movies 12-14). Bars, 100 μm.

migration. This situation could be considered analogous to that seen in many cell types which express *cxcr*4b (e.g. cells in the somites and along the pronephric duct) yet are indifferent to SDF-1a (Chong et al., 2001; Doitsidou et al., 2002). These are the PGC-specific steps of maturation, culminating in migratory competence, that differentiate these cells from the surrounding CXCR4b-expressing somatic cells. Determining the molecular basis underlying these alterations is likely to provide insights into processes involving similar transitions. For example, the transition observed in PGCs may shed light on the process of epithelial-mesenchymal transition where cells loose their epithelial properties and assume 'amoeboid' invasive migratory behaviour (Savagner, 2001; Thiery, 2002).

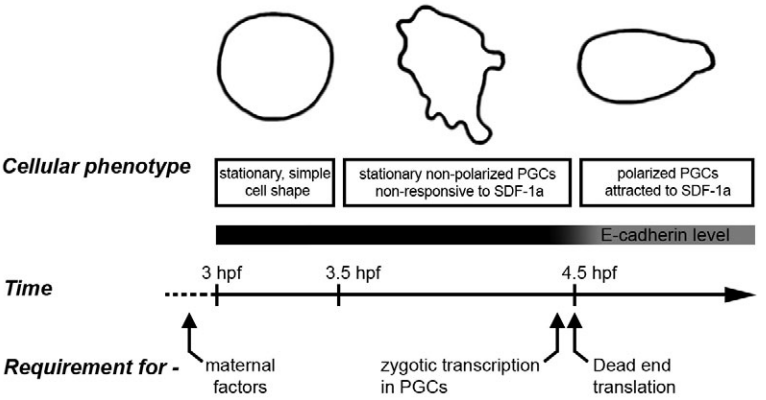

Fig. 6. Differentiation stages during early PGC development leading to motility, polarization and guided migration. Following their specification, a process which is controlled by maternally provided germ plasm (Hashimoto et al., 2004; Knaut et al., 2000; Yoon et al., 1997), the PGCs appear morphologically similar to somatic cells, yet they show expression of characteristic markers and can be specifically labelled with GFP. During the next stage, the PGCs assume a complex non-polarized morphology, express high levels of E-cadherin on their membrane and do not respond to guidance cues provided by the chemokine SDF-1a. Further differentiation depends on de novo transcription in the zygote since inhibition of transcription by α-amanitin leads to a complete developmental arrest of PGCs at the second phase. The transition to the third stage relies on the function of the Dead end protein. This stage is characterized by moderate down regulation of E-cadherin and the competence of PGCs to polarize and migrate in response to directional cues.

Discussion

The results presented in this work demonstrate that following their specification, zebrafish PGCs undergo a string of distinct differentiation steps that culminate in their migration, summarized in (Fig. 6). This description of the onset of zebrafish PGC migration may represent a general behaviour of cells before they become migratory. For example, studies of live *Drosophila* PGCs isolated and cultured in vitro show striking similarities to the behaviour of zebrafish PGCs in vivo: shortly after their specification, cultured *Drosophila* PGCs are not motile and subsequently, they randomly extend small protrusions followed by pseudopodia formation and cell polarization (Jaglarz and Howard, 1995). Remarkably, neural crest cells delaminating from the neural tube exhibit a behaviour that is qualitatively similar to that described above for zebrafish PGCs early in their development.

Here, the cells initially display excessive membrane blebbing that is followed by extension of lamellipodial processes and movement away from the neural tube (Halloran and Berndt, 2003). The step-wise manner by which cells acquire their competence to migrate appears to pertain to abnormally developing cells as well, since a subset of primary tumour cells that can polarize in the direction of blood vessels show increased metastatic potential (e.g. Wyckoff et al., 2000). Whereas this transition phase is an essential first step for cell migration, it is poorly understood at the molecular level. Specification of the migratory cell fate in the case of border cells depends on the function of the JAK-STAT pathway (Beccari et al., 2002; Silver and Montell, 2001), while neural crest cell migration depends on proteins of the Snail family (Nieto, 2001). Nevertheless, the molecules that are directly involved in the transition are unknown. Considering that PGC transition to a polarized directed migration requires functional Dead end, a sound strategy for identifying these molecules would be to use Dead end as a tool. Isolating RNA molecules that interact with the RNA-binding protein Dead end is likely to uncover genes that play a role in the transition of PGCs to guided active migration. Additionally, important clues can be obtained from microarray-based analysis comparing the transcriptom of metastatic cells with that of non-metastatic cells. In these studies a dramatic up regulation of genes that are part of the 'minimum motility machine', such as genes encoding cofilin, capping proteins and components of the Arp2/3 pathways, are observed (e.g. Wang et al., 2004). However, the precise role of these proteins in initiating the motile behaviour is not clear. Studying the function of proteins involved in metastasis in the context of zebrafish PGC migration is therefore an attractive avenue for determining the role they play in metastasis and in normal development.

Previous studies of PGC migration have identified CXCR4b and its ligand SDF-1a as the receptor-ligand pair guiding the cells to their targets (Doitsidou et al., 2002). CXCR4b however, is likely to be irrelevant to the control of the initiation of PGC migration. First, *cxcr4b* RNA is maternally provided (Chong et al., 2001) (our unpublished results) and thus could not be the limiting factor necessary for the transition to a motile behaviour. A second possibility is that *cxcr4b* RNA translation could represent a step during early PGC maturation that would provide functional CXCR4b protein by the time the cells start migrating towards SDF-1a. However, the finding that in the absence of functional CXCR4b, the PGCs actively migrate, albeit non-directionally (Doitsidou et al., 2002; Knaut et al., 2003), would argue against this scenario. A more likely group of molecules that could control PGC motility in zebrafish is suggested by the demonstration that the migration speed and cell morphology are controlled by PI3K (Dumstrei et al., 2004). Monitoring the activity of this molecule, the activity of molecules acting downstream of it (e.g. Rho GTPases) and those constituting the 'minimum motility machine' during early PGC development is expected to provide an insight into the mechanism underlying the transition from immotile to motile cell behaviour.

As PGCs in different organisms appear to utilize similar molecular pathways for their migration (Molyneaux and Wylie, 2004; Raz, 2004; Santos and Lehmann, 2004), the results described here may be relevant for mouse and *Drosophila* PGCs, as well. The finding that early zygotically transcribed genes and those whose function is regulated by the Dead end protein are essential for PGC transition from non-motile to directionally migrating cells should allow focusing the search for the specific molecules involved in this process.

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