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Sequential SDF1a and b-induced mobility guides Medaka PGC migration

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ABSTRACT

Assembly and formation of the gonad primordium are the first steps toward gonad differentiation and subsequent sex differentiation. Primordial germ cells (PGCs) give rise to the gametes that are responsible for the development of a new organism in the next generation. In many organisms, following their specification the germ cells migrate toward the location of the prospective gonadal primordium. To accomplish this, the PGCs obtain directional cues from cells positioned along their migration path. One such cue, the chemokine SDF1 (stromal cell-derived factor 1) and its receptor CXCR4 have recently been found to be critical for proper PGC migration in zebrafish, chick and mouse.

We have studied the mechanisms responsible for PGC migration in Medaka. In contrast to the situation observed in zebrafish, where proper PGC positioning is the result of active migration in the direction of the source of SDF1a, Medaka PGC movements are shown to be the consequence of a combination of active SDF1a and SDF1b-guided migration. In this process both SDF1 co-orthologues show only partly overlapping expression pattern and cooperate in the correct positioning of the PGCs.

Introduction

Gonad assembly and formation are the first steps toward gonad differentiation and subsequent sex differentiation (Camerino et al., 2006; Werner et al., 1996). Similar to other vertebrates, the structure of fish gonads is composed of germ cells and associated supporting somatic cells (Delvin and Nagahama, 2002). The precursors of the somatic cells originate from cells of the paraxial mesoderm where the gonadal primordium develops, while germ cells are derived from the germline lineage.

In mouse and mammals primordial germ cells are induced from the pluripotent epiblast cells before and during gastrulation by extraembryonic cell-derived signals (mainly BMPs) (Matsui and Okamura, 2005; McLaren, 2003; Raz, 2005). In contrast, for many invertebrate and nonmammalian vertebrate species, PGC development is characterized by the inheritance of germ plasm, a cytologically distinct assembly of mitochondria and electron-dense germinal granules (Jin and Xie, 2006). In Medaka we provided evidence that very early during development, PGCs specification relied on germ plasm and was independent of induction processes from the surrounding somatic tissue (Herpin et al., 2007a).

Once specified and segregated from somatic tissues–as early as beginning of gastrula (stage 14/15)–Medaka PGCs have to migrate from the position where they were specified toward the region where the prospective primordial gonad will form and develop. Hence, from the initial scattered configuration at the early gastrula stage, the PGC move to the marginal zone of the embryonic shield. During neurulation and early somitogenesis (stage 15 to 23) PGCs converge toward the midline along the body axis. By stage 24 they reach the lateral plate mesoderm and then cluster in the area of the prospective primordial gonad (Herpin et al., 2007a,b; Kurokawa et al., 2006).

To reach their target, the PGCs obtain directional cues from cells positioned along their migration path. One such cue, the chemokine SDF1 (stromal cell-derived factor 1) and its receptor CXCR4 have recently been found to be critical for proper PGC migration in zebrafish and mice (Ara et al., 2003; Doitsidou et al., 2002; Molyneaux et al., 2003). SDF1 is expressed in somatic cells while the seven transmembrane G protein-coupled receptor of SDF1a, CXCR4, is expressed in germ cells (Knaut et al., 2003). Interestingly, in Medaka, in addition to SDF1b (Kurokawa et al., 2006), SDF1a, a second putative ligand for CXCR4 (Yasuoka et al., 2004) has been identified.

In this work we investigated the mechanisms regulating PGC migration in medaka. In contrast to the situation observed in zebrafish where proper PGC positioning is the result of a gradual acquisition of responsiveness to SDF1a followed by active migration toward the source of the chemokine, PGC movements in medaka were found to be

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the consequence of alternation of active SDF1a and SDF1b-induced directed migration and possible passive movement along with neighboring somatic cell movements. This study points out the complementary roles of the two SDF1 duplicates and shows that a similar developmental process may be fine-tuned differently in different fish species.

Materials and methods

Vector preparation, RNA and morpholino embryo injections

Capped RNA for injections was transcribed from linearized vectors using the SP6 mMESSAGE mMACHINE Kit (Ambion). One nanoliter was injected into the cytoplasm of one-cell stage Medaka embryos as described (Koster et al., 1997). For knockdown experiments, embryos were injected with SDF1-W: 5′-TGAGCGCAAAGAGCTTCACATC-CAT-3′ and SDF1-T: 5′-TCAGTGCGAGCAGCTTGGCGTCCAT-3′ against either the Sdf1a or the Sdf1b ATG region and with Sp1Sdf1a: GAGCATTTTAAAGACTTACCGTGTG, Sp2Sdf1a: TTGCTGCAAAGGAAAGATGAATAGC and Sp1Sdf1b: GGTGTCCCTGGACTCACCTTTGGAG, Sp2Sdf1b: TTTGGCTGCAAGACACAAACCGCAG against the intron 1 of either Sdf1a or Sdf1b. The most efficient doses (3.5 mg/mL) were experimentally determined and the specificity of the oligos confirmed in control experiments (see Supplemental Figs. 2A to H). As controls for morpholino induced phenotypes splicing morpholinos against Sdf1a and Sdf1b intron 1 were purchased (Supplemental Figs. 2E to H).

The *gfp-nos1* 3′UTR construct includes the mmGFP5 ORF cloned upstream of the 3′ UTR of the zebrafish nanos1 gene (Koprunner et al., 2001).

The *sdf1a-nos1* 3′UTR construct was prepared by replacing GFP by Medaka *sdf1a* ORF (*Bam*H1/*Eco*R1 sites) in the above described *gfp-nos*1 3′UTR construct.

In situ hybridization

One- and two-color RNA whole-mount *in situ* hybridization using *sdf1a* and *sdf1b* (Kurokawa et al., 2006) DIG/FLU labeled probes were performed as previously described (Winkler et al., 2003). Staining time was individually adjusted for each probe to get the best signal and does not reflect the endogenous transcript expression level.

Experimental animals

Medakas (*Oryzias latipes*) of strain Carbio (*cab*) were used in this study. The fish were maintained essentially as described for zebrafish (Westerfield, 1995). Developmental stages were determined according the criteria set out by Iwamatsu (2004).

Molecular phylogeny

Predicted amino acid sequences were aligned automatically by T-Coffe (Notredame et al., 2000). Phylogenies were determined with PAUP* (D.L. Swofford, Smithsonian Institution, WA, DC) by bootstrap analysis using maximum parsimony (1000 replicates) and neighbor-joining (Saitou and Nei, 1987).

Expression analyses

Total RNA was extracted from Medaka embryos at different stages of development (stages 3/4, 8, 9/10, 12, 13, 14/15, 16, 18, 20, 24, 25, 33, 36 and 39 according to Iwamatsu, 2004) using Total RNA Isolation Reagent (ABgene) according to the supplier's recommendation. After DNase treatment, reverse transcription was performed with 2 μg total RNA using RevertAid First Strand Synthesis kit (Fermentas) and oligo dT primer. Real-time quantitative PCR was carried out with SYBR Green/FITC reagents and amplifications were recorded with an i-Cycler (Biorad). All results are averages of two independent RT reactions and 3 PCR reactions from each RT reaction. Relative expression levels (according to the equation $2^{-\Delta CT}$) were calculated taking elongation factor 1α (*ef1α*) as a reference.

Results and discussion

Isolation of two SDF1 co-orthologues in medaka

To examine the role of SDF-1 in PGC migration in medaka, we cloned and characterized a second Sdf1 gene in Medaka.

To establish the evolutionary relationship of the medaka SDF1 sequences a phylogenetic tree of vertebrate SDF1s was constructed (Fig. 1A). The overall topology of the tree clearly defines a teleost clade separated from other vertebrate SDF1 sequences. Within teleost sequences, two branches can be clearly distinguished, namely “a” and “b”. This identifies clearly the previously described SDF1a (Kurokawa et al., 2006) as a “b” form whereas the newly described SDF1 is nested with the SDF1a clade. Thus the “SDF1a” described by Kurokawa et al. (2006) has to be renamed SDF1b. The molecular phylogenetic analysis also revealed that the two Medaka Sdf1 genes are co-orthologues of a unique tetrapod Sdf1, suggesting that teleost Sdf1a and Sdf1b likely arose from the teleost specific whole genome duplication (Meyer and Schartl, 1999; Postlethwait et al., 2004) and have a common ancestor.

To this point, linkage mapping analysis for both Sdf1a and Sdf1b in Medaka and zebrafish supports this idea (see Supplemental Fig. 1). Medaka and zebrafish Sdf1a are located on linkage group OlaLG15 and DreLG13 respectively, while Medaka Sdf1b was mapped to OlaLG1 and zebrafish Sdf1b is located on DreLG22. LG1 and LG15 of Medaka and zebrafish LG13 and LG22 display strong synteny to each other as well as to human chromosome 10 on which the human Sdf1 is located (see Supplemental Fig. 1). Taken together these data strongly support the hypothesis that the teleost Sdf1 gene duplicates arose from the teleost specific whole genome duplication and thus are clearly co-orthologues. Finally, the fact that neoteleostei (Medaka, takifugu and tetraodon) SDF1a are not nested within the cypriniform SDF1a clade-like observed for the “b” form (Fig. 1A), likely reflects different evolutionary rates between neoteleostei “a” forms.

Embryonic expression of SDF1a and SDF1b

To clarify the importance and functional roles of the two SDF1 co-orthologues during early primordial germ cell migration, the temporal expression pattern of the “a” and “b” forms was investigated. Different from the receptor CXCR4, which is maternally expressed (Kurokawa et al., 2006), both *sdf1a* and *sdf1b* RNAs are not deposited to the oocyte but are zygotically transcribed from stage 10 (mid-blastula) of development onwards (Kurokawa et al., 2006 and Fig. 1).

For a more quantitative analysis regarding the relative expression of the two Sdf1 co-orthologues, real-time PCR quantification of the two *sdf1* transcripts was performed (Figs. 1C and D). Interestingly, the *sdf1a* and *sdf1b* expression kinetics (Figs. 1C and D) are quite different. The preferential *sdf1a* expression during early development up to the beginning of somitogenesis (stage 20) is arguing for a major early contribution of Sdf1a (Fig. 1C). The dramatic reduction of *sdf1a* expression from stage 24 (somitogenesis) onward and the simultaneous rise of *sdf1b* suggest that this molecule functions during later stages of development (Fig. 1D). Noteworthy, by stage 39, when the primordial gonad has already assembled, the second rise of *sdf1a* could reflect other functions that do not have to do with germ cell migration such as pigment cell patterning (Svetic et al., 2007).

In addition, the spatial expression pattern for both *sdf1s* becomes quite divergent during development (Fig. 2). *In situ* hybridization revealed that early on, from the onset of gastrulation (stage 15) to the end of neurulation (stage 18), expression of the two co-orthologues coincides at the marginal zone with progressive restriction of the expression to the dorsal region of this marginal zone (Kurokawa et al., 2006; Nakamura et al., 2006 and Figs. 2A to D). At the end of neurulation (stages 18/21) restricted expression of both *sdf1s* is observed in the forming lateral plate mesoderm (Kurokawa et al., 2006 and Figs. 2C to F). Noteworthy, by stage 24 during segmentation, *sdf1a* and *sdf1b* expression patterns become distinct from one another. *sdf1a* is exclusively expressed in the newly formed somites (Figs. 2G–I) and *sdf1b* in the lateral plate mesoderm/presomitic mesoderm (Fig. 2H). Hence, during early development up to completion of neurulation, the expression patterns of the two Sdf1 co-orthologues coincide with the stage-specific localization of Medaka PGC (Herpin et al., 2007a).

In summary, the expression data suggest that although coincidently expressed during early PGC migration, a more prominent contribution can be attributed to the highly expressed *sdf1a* orthologue, while during somitogenesis *sdf1b* being specifically expressed in the lateral plate mesoderm (where the PGCs are located

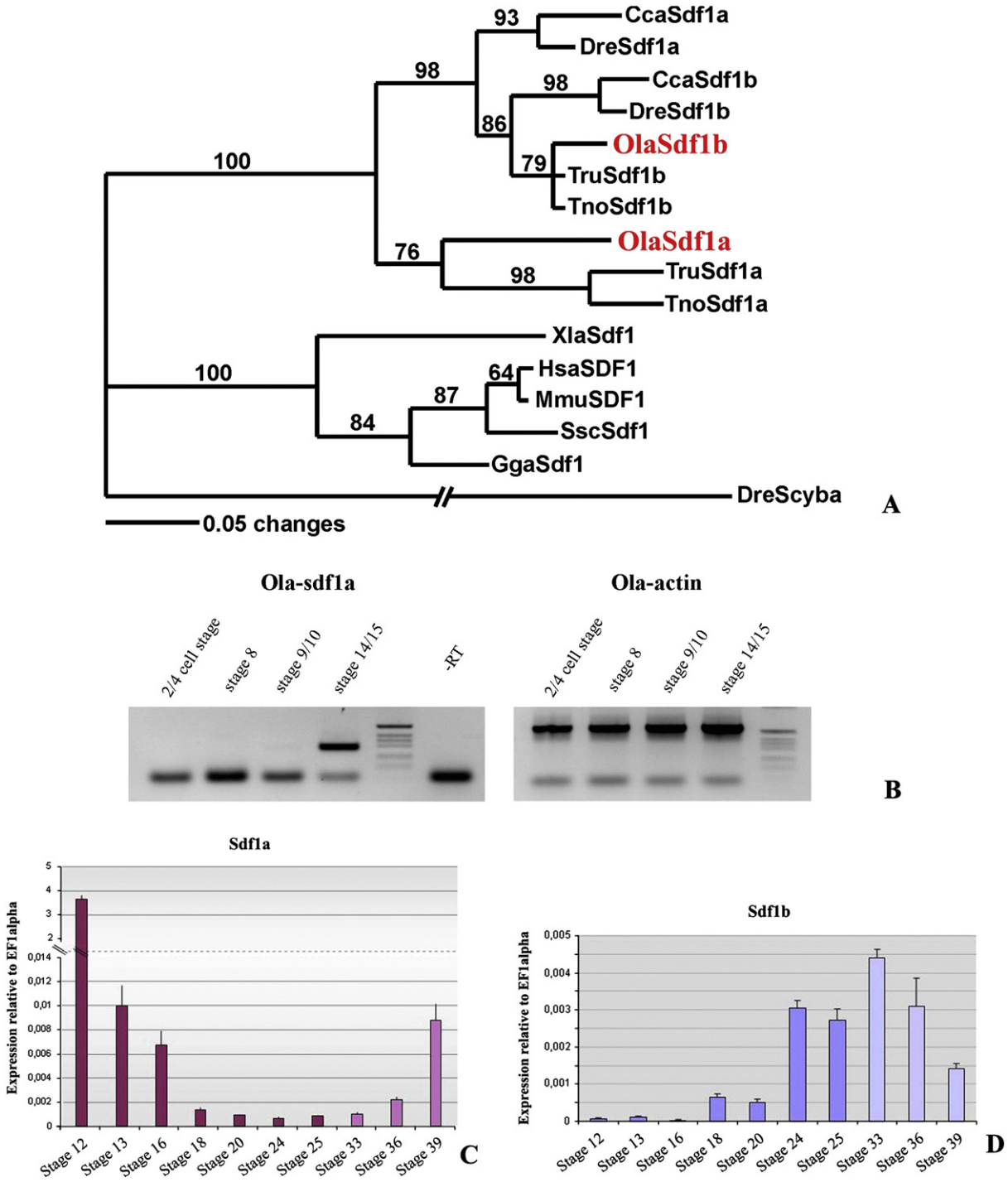

Fig. 1. Phylogeny and divergent expression patterns of SDF1 duplicates. (A) Neighbor-joining tree of the Sdf1 CXCL12 chemokine family. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Accession numbers are the following: SDF1a *Cyprinus carpio* (CcaSdf1a; CAF28458), SDF1b *C. carpio* (CcaSdf1b; CAD59916), SDF1a *Danio rerio* (DreSdf1a; AAS92649), SDF1b *D. rerio* (DreSdf1b; AAQ24225), SDF1 *Gallus gallus* (GgaSdf1; AAR88102), SDF1 *Homo sapiens* (HsaSDF1; P48061), SDF1 *Mus musculus* (MmuSDF1; P40224), OlaSDF1a *Oryzias latipes* (BAE92943), OlaSDF1b (sdf1a) *O. latipes* (DQ859774), SDF1 *Sus scrofa* (SscSdf1; AAZ32768), SDF1a *Takifugu rubripes* (TruSdf1a; SCA 24), SDF1b *T. rubripes* (TruSdf1b; SCAF 26), SDF1a *Tetraodon nigroviridis* (TnoSdf1a; Chr 17 Scaf 15006), SDF1b *T. nigroviridis* (TnoSdf1b; Chr 18), SDF1 *Xenopus laevis* (XlaSdf1; NP_001083632). Outgroup: CXCL14 *D. rerio* (DreScyba; AAG09819). OlaSDF1a clusters within the SDF1b clade of teleosts, whereas OlaSDF1 is nested within the SDF1a clade. (B) Early expression (PCR) of *sdf1a.* No transcripts are detectable before mid-blastula transition (stage 9/10) when zygotic transcription starts. Quantitative determination of expression of *sdf1a* (C) and *sdf1b* (D) by real-time PCR.

at this stage) is likely the prominent factor with respect to PGC localization. Consequently, we can reasonably assume that Medaka PGC guidance is achieved by joint–and probably redundant–action of SDF1a and SDF1b during early PGC migration (when both are coincidently expressed), while a prime role could be exerted by SDF1b when segmentation initiates.

SDF1a and SDF1b co-orthologues are alternatively involved during primordial germ cell migration

The possibility to observe *in vivo* PGC behavior at the earliest stages of their development (Herpin et al., 2007a) by GFP expression controlled by the *nos3′UTR* allowed us to investigate the direct

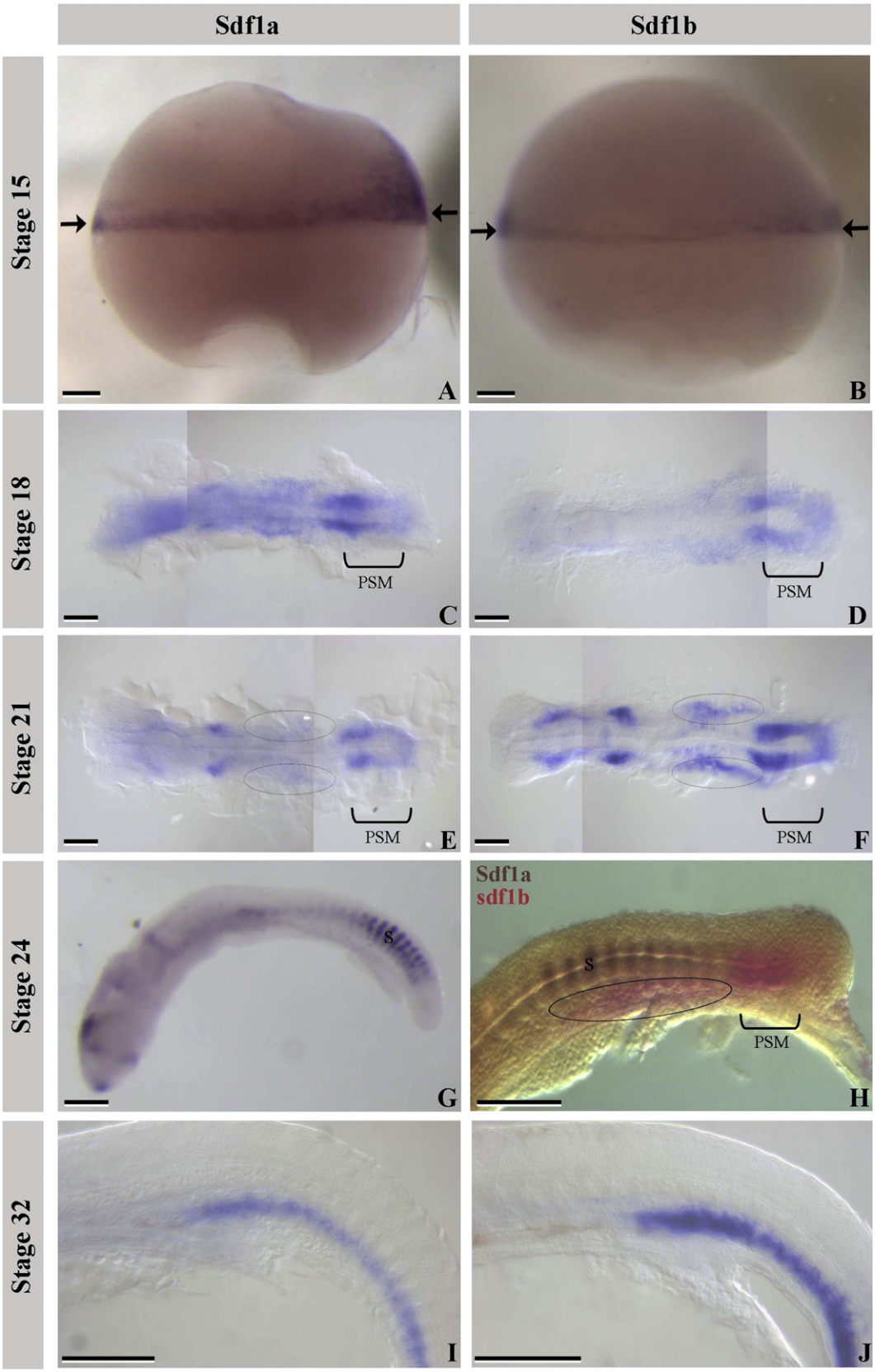

Fig. 2. Divergent spatial expression patterns of Sdf1 duplicates. (A to J) *sdf1a* and *sdf1b* transcripts were detected by whole-mount *in situ* hybridization. From the onset of gastrulation (stage 15) to the end of neurulation (stage 18) expression of the two sdf1 co-orthologues coincides at the marginal zone with progressive restriction of the expression to the dorsal region of the marginal zone (A and B). Subsequently, for the duration of early segmentation (stages 18 to 21), *sdf1a* and *sdf1b* expression areas are restricted to the lateral plate and presomitic mesoderm (C–F). By stage 24, during segmentation, *sdf1a* and *sdf1b* spatial expression patterns become divergent with expression of *sdf1a* in the newly formed somites (G and I) and *sdf1b* in the lateral plate mesoderm/presomitic mesoderm respectively (H and J). The dotted circles indicate the lateral plate mesoderm (LPM) while black arrows indicate the marginal zone of the embryo. Brackets set the limits of the presomitic mesoderm area; (S) somites. Scale bar, 110 μm.

contribution of each Sdf1 co-orthologue individually. We aimed at reducing Sdf1a and Sdf1b activity using orthologue-specific modified antisense oligonucleotides (morpholinos), either translation-blocking or splicing-blocking morpholinos.

Sdf1a knockdown

In order to examine whether Sdf1a is indeed mainly required for early PGC guidance just after their specification, translation and splice-blocking morpholinos against *sdf1a* together with *gfp-nanos* 3′ UTR mRNA were injected in medaka embryos at the one-cell stage and the PGC migration path was monitored (Fig. 3 and Supplemental Fig. 3). The onset of PGC specification was monitored by expression of *GFP:nos3′UTR* mRNA (Herpin et al., 2007a). Already at that stage PGCs of *sdf1a* morphant embryos did not reach the dorsal marginal zone like in control embryos but remained rather scattered around the animal pole (Fig. 3D compared to A and Supplemental Figs. 3A and B). Thereafter, by the completion of neurulation, those PGCs remaining at the shield region close to the anteroposterior axis got closer to the embryonic body, although distributed all along the whole axis and not concentrated at its posterior part as in controls (Figs. 3E, F and P compared to Figs. 3B, C and M and Supplemental Fig. 3D and E).

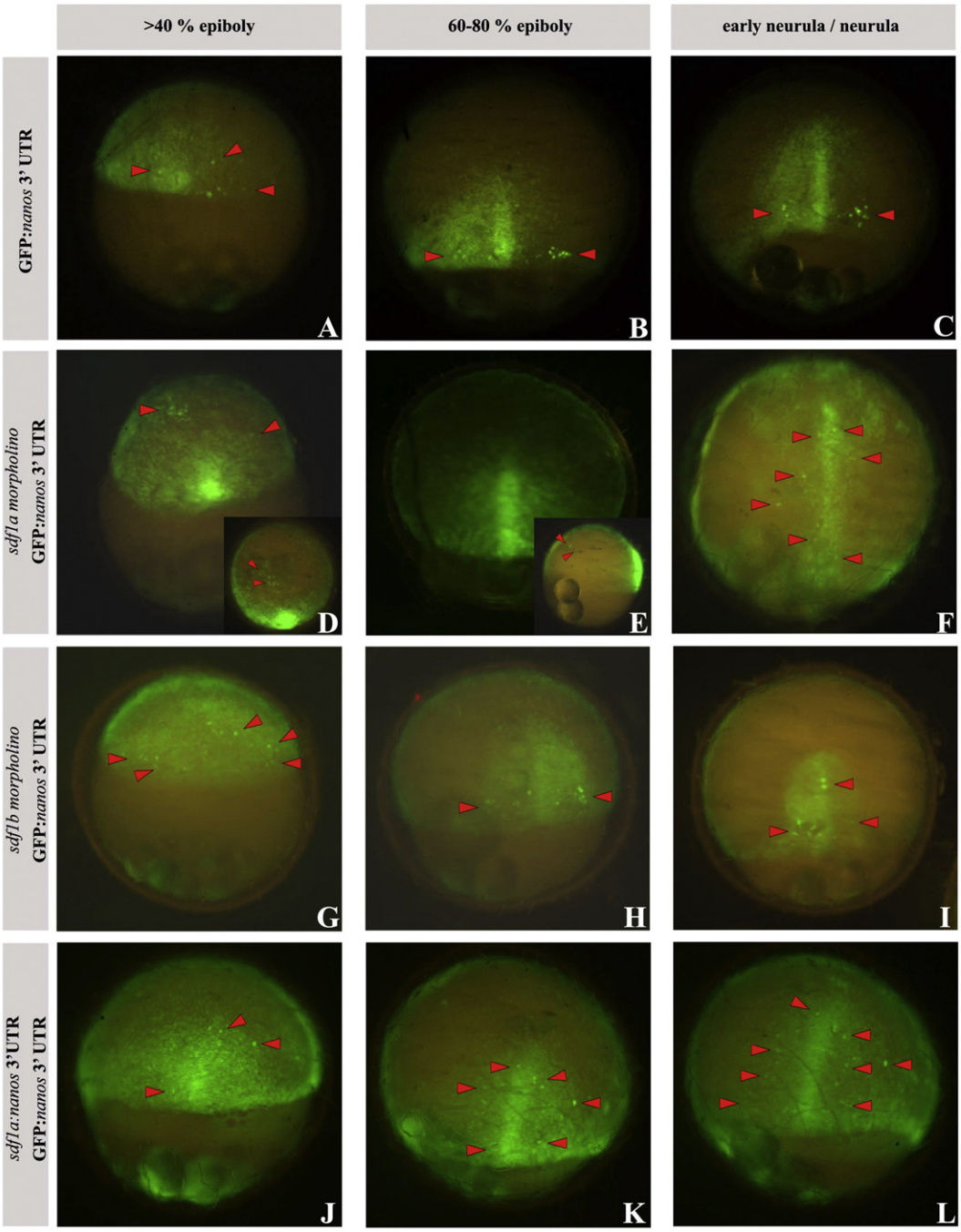

Fig. 3. Impaired PGC migration after translation-blocking *sdf1a*-MO and *sdf1b*-MO injection or blocking of *cxcr4*. Embryos coinjected with *gfp:nanos* 3′UTR and either Sdf1a-MO- or Sdf1b-MO-morpholinos or *sdf1a:nanos* 3′UTR were compared for PGC migration during early development: 40% epiboly (A, D, G, J), 80% epiboly (B, E, H, K), early neurula (C, F, I, L), late neurula (M, P, S, V), 15 somite stage (N, Q, T, W) and 22 somite stage (O, R, U, X). The different insets display impaired PGC distribution like observed for top view (D), lateral view (E, P) and head magnification (R, T) of the embryos.

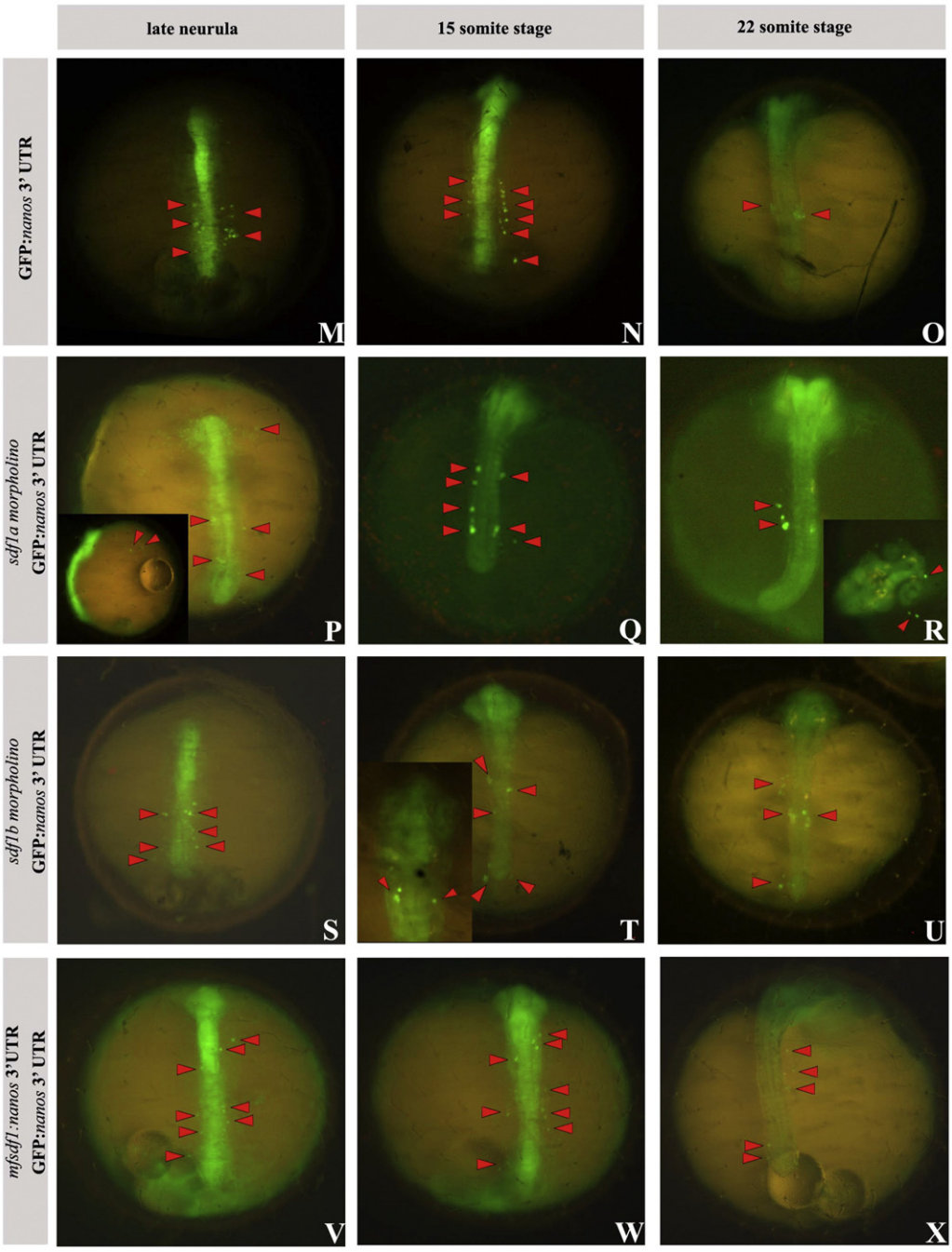

Fig. 3 (*continued*).

During somitogenesis those PGCs present in the lateral plate mesoderm area regrouped and clustered as in control embryos (Figs. 3Q and R compared to Figs. 3N and O and Supplemental Fig. 3G, H, J and K). At this stage some PGCs were seen spread all over the yolk sac epithelium, even diametrically opposed to the embryonic body (see detail in Figs. 3Q and R and schematic representation in Fig. 4).

Sdf1b knockdown

Contrary to *sdf1a* morphant embryos, *sdf1b* morpholino-injected embryos did not exhibit PGC migration abnormalities at early stages of development (Figs. 3G–I and Supplemental Fig. 3C). After specification at blastula stage PGCs accumulated around the peripheral margin and then moved progressively to the dorsal part of this zone (Figs. 3G and H and Supplemental Fig. 3C). Thereafter, from stage 16 to 18 (neurula), PGCs began to cluster around the posterior part of the embryonic body (Fig. 3I and Supplemental Fig. 3F). The first apparent difference to control embryos was observed at the time of migration from the tail bud region toward the lateral plate mesoderm around stage 24/26. Most of the PGCs remained in the posterior lateral plate mesoderm (in agreement with observations by Kurokawa et al., 2006). They did not reach the prospective primordial gonad (Figs. 3S–U compared to Figs. 3M–O and Supplemental Fig. 3F, I and L). Fig. 4 provides a schematic representation of PGC localization after morpholino injections.

Although the use of either *sdf1* translation-blocking or *sdf1* splice-blocking morpholinos resulted in the same impaired PGC migration phenotypes (Fig. 3 compared to Supplemental Fig. 3), interestingly

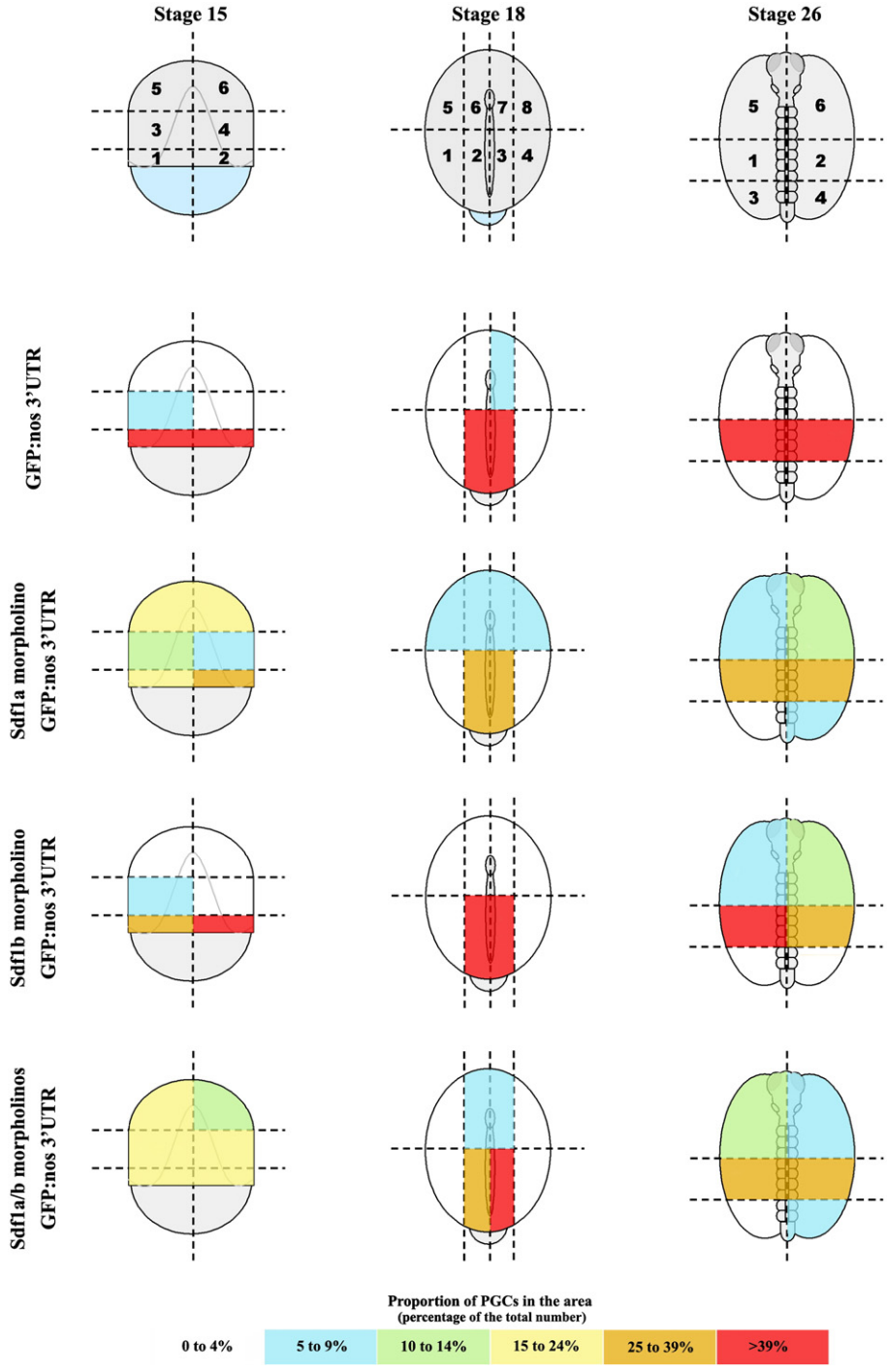

Fig. 4. Schematic representation of impaired PGC migration after morpholino knockdown. Embryos were coinjected with *gfp*:*nanos* 3′UTR and either translation-blocking Sdf1a-MO or Sdf1b-MO or both (double knockdown). For each area (1 to 8) PGCs were counted at different stages of development (stage 15 mid-gastrula, stage 18 early neurula, stage 26 22 somite stage). Control embryos were injected with only *gfp*:*nanos* 3′UTR. Raw data for the percentages are given in Supplemental Fig. 5.

splice morpholinos allowed us to evaluate to what extent the activities of SDF1a and SDF1b were blocked (Supplemental Figs. 2E to G). It revealed that down-regulation of 30% or more (Supplemental Fig. 2) was enough to suppress *sdf1* gradients that normally guarantee proper PGC migration.

Double knockdown of Sdf1a and Sdf1b

To detect possible synergy between the SDF1 duplicates, *sdf1a* and *sdf1b* were knocked down simultaneously and the phenotype was compared to the single knockdowns (Fig. 4). Although to some extent more mislocalized PGCs could be observed for SDF1 double knockdown embryos at stage 15 when compared to single *sdf1a* or *sdf1b* morphant embryos, by stage 26 similar PGC distribution pattern could be observed. For these reasons, if redundant actions of Sdf1a and b are likely to occur between the two co-orthologues during the early PGC migration phase (before stage 15), any synergistic effect can be ruled out at later stage (Fig. 4). Nevertheless, relative SDF1a and SDF1b affinities against CXCR4 receptor remain to be investigated in order to draw a robust conclusion.

Specific inhibition of Sdf1a-mediated signaling in PGCs

Proper PGC migration and guidance are achieved through specific and temporal synexpression of SDF1a/b proteins in somatic tissues and CXCR4 in the PGCs (Raz, 2003; Raz and Reichman-Fried, 2006; Fig. 5).

In order to analyze the relevance of chemotactic cues that guide PGC migration, the capability of SDF1a receptor(s) to transmit a polarized signal was eliminated specifically in the PGCs by injecting *sdf1a:nanos* 3′UTR mRNA. A primordial germ cell specific expression of SDF1 should saturate the SDF1a-mediating receptor(s) and therefore make the cells unresponsive to the polarized guidance by SDF1 expressed by the somatic environment (Doitsidou et al., 2002). Coinjection with *gfp-nanos* 3′UTR was performed to observe PGC migration and to compare this to control *gfp-nanos* 3′UTR only injected Medaka embryos (Figs. 3J–L, V–X). Although less severe than in *sdf1* morpholino-injected embryos, PGC migration was similarly affected at different stages of development (Figs. 3J–L, V–X).

(I) Between 40% epiboly up to early neurula, contrary to the normal situation, no migration of PGCs to the marginal zone was seen for *sdf1a:nanos* 3′UTR injected embryos. This indicates that this first step of early PGC migration is highly dependent on SDF1a and CXCR4 (Figs. 3J, K and L) function. Saturation of SDF1a receptor(s) by SDF1a overexpression totally abolished SDF1-induced active PGC migration. This effect results in a similar outcome as seen in *sdf1a* morphant embryos. Noteworthy, injection of a morpholino directed against *sdf1b* did not affect PGC migration at this early stage (Kurokawa et al., 2006), suggesting a specific involvement of the SDF1a/CXCR4 ligand–receptor pair during early PGC migration.

(II) Between late neurula and up to the 15 somite stage, in both injected and control embryos, PGCs migrated toward the midline along the body (Figs. 3V and W). This is in agreement with observations by Kurokawa et al. (2006), that *sdf1b* morphants did not display any apparent defects in PGC migration up to these stages. Because neither Sdf1a-overexpressing (CXCR4 receptor saturated) nor *sdf1a/b* knocked down embryos displayed impaired PGC migration behavior between neurula and 15 somite stage, PGC movements toward the embryonic body are likely to be uncoupled with SDF1a, SDF1b and CXCR4 actions (Figs. 3V and W). Although other active mechanisms could not be ruled out, our findings are consistent with the idea that PGCs passively converge along with somatic cells (Kurokawa et al., 2006).

(III) Around the 22 somite stage, most of the PGCs expressing *sdf1a::nanos* 3′UTR did not reach the posterior portion of the lateral plate mesoderm and did not move at all (Fig. 3X). This infers an active posterior migration toward the posterior lateral plate mesoderm, which is highly dependant of Sdf1b, being expressed there.

Conclusion

In several vertebrates including zebrafish, chick and mouse, recent studies have shown that early PGC migration and later on PGC guidance toward the posterior lateral plate mesoderm, where the primordial gonad is developing, depends on the activity of the chemokine receptor CXCR4 and its SDF1a ligand (Doitsidou et al., 2002; Molyneaux et al., 2003; Stebler et al., 2004). The possibility to observe *in vivo* PGCs at the earliest stages of their development allowed to investigate the progression of the cellular response to external cues, namely to SDF1a and SDF1b through binding to the CXCR4 receptor (Doitsidou et al., 2002; Kurokawa et al., 2006; Yasuoka et al., 2004).

Noteworthy, although mainly overlapping with *sdf1b* (Kurokawa et al., 2006), *sdf1a* expression pattern displayed some specific characteristics, possibly explaining the different and overlapping functions of both factors described above. The identical spatial expression patterns during early gastrulation would suggest a partially redundant or similar action of SDF1a and SDF1b. However, although relative SDF1a and SDF1b affinities against CXCR4 receptor remain to be investigated in order to draw a robust conclusion, real-time PCR data showing predominant expression of *sdf1a* orthologue before somitogenesis would place SDF1a as the major player for this early phase. Next, during early somitogenesis a broader and weaker expression of *sdf1a* and *b* respectively was observed along the lateral plate mesoderm. In accordance with Kurokawa et al. (2006) observations, and consistent with the idea that the PGCs passively converge along with somatic

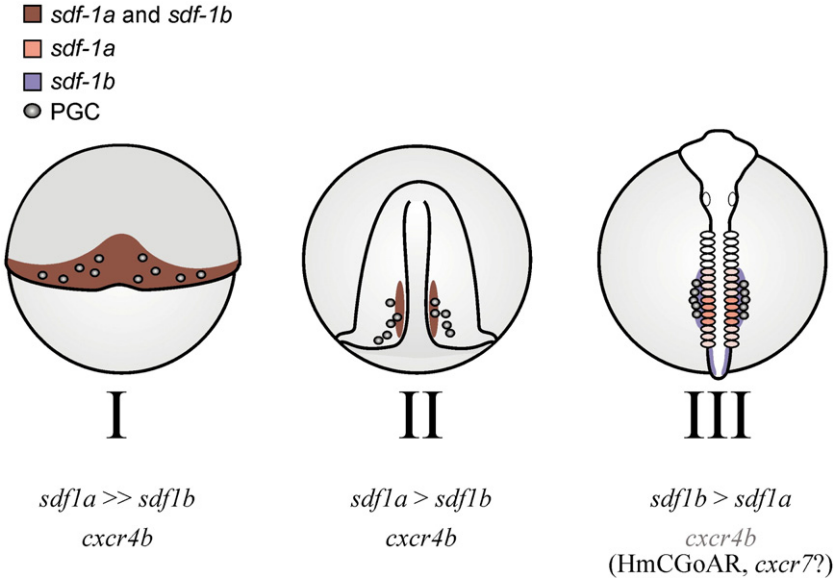

Fig. 5. PGC migration in Medaka is the consequence of alternation of active OlaSDF1a/b-induced mobility. During the process of early PGC migration (Step I), CXCR4 and Sdf1a are absolutely required for proper PGC migration. Next, during neurulation/early somitogenesis (Step II), PGC migration is possibly dependant of somatic movements, while arrest of PGCs in the lateral plate mesoderm along the embryo body is possibly due to SDF1a action. Finally, during late PGC migration (step III), the role of Sdf1b is becoming predominant (Kurokawa et al., 2006) while the function of Sdf1a and CXCR4 although still required appears to be much less important. The recent description of the sequential action of the CXCR4 and CXCR7 receptors in regulating PGC migration in Medaka (Sassado et al., submitted), indicates that a CXCR7-dependent process would act after the CXCR4-dependent bilateral alignment of PGC and causes drift of PGC to the gonadal area.

cells, the observed accumulation and arrest of the PGCs next to the embryonic body close to the lateral plate mesoderm location are likely to be independent of SDF1a, SDF1b and CXCR4 actions. This clustering to the anterior region of the lateral plate mesoderm is paralleled by a marked restriction of *sdf1b* expression to this region and a strong up-regulation of transcript levels (Kurokawa et al., 2006). The exclusive expression in the posterior somites makes any participation of SDF1a during this late process of PGC migration unlikely.

Finally, our results inhibiting SDF1a-mediated signaling in PGCs combined with the specific *sdf1a/sdf1b* expression patterns, support a redundant activity of SDF1a and SDF1b with regard to CXCR4 binding during early PGC migration and speak against likely any synergistic effects between the two duplicates at later stages.

The recent description of the sequential action of the CXCR4 and CXCR7 receptors in regulating PGC migration in Medaka (Sassado et al., submitted), indicates that a CXCR7-dependent process would act after the CXCR4-dependent bilateral alignment of PGC and causes drift of PGC to the gonadal area. The overlap of the expression domains of *sdf1a*, *sdf1b* and *cxcr7* combined with the observations that *sdf1b* knockdown (Fig. 3 and Supplemental Fig. 3 and Kurokawa et al., 2006) as well as inhibition of CXCR4 receptor interfered with posterior migration of the PGCs would be consistent with a model that SDF1a and SDF1b, in addition to CXCR4 also act as ligands of CXCR7 in the somites. In addition, while evidence consistent with the idea that CXCR7 would act as a nonsignaling receptor that functions as a sink for SDF1a has been provided in the case of zebrafish PGC migration (Boldajipour et al., 2008), its activity seems to be essential for attaining a distribution of SDF1a that is capable of polarizing the PGCs and directing their migration toward cells expressing the RNA of their attractant (Boldajipour et al., 2008).

Hence, as a conclusion, with respect to the process of subfunctionalisation described for teleost duplicated genes (Meyer and Schartl, 1999; Postlethwait et al., 2004), it is likely that the ancestral function of mammal-type sdf1 gene in PGC migration guidance is, in teleost and *a fortiori* in Medaka, endorsed by both SDF-la and SDF-1b co-orthologues binding with cell type specific affinities to one (CXCR4) or more (CXCR7) receptor type(s) in order to regulate the fine tuning of general SDF1 gradient.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.03.030.

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