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Expression of *xSDF-1α, xCXCR4* and *xCXCR7* during gastrulation in *Xenopus laevis*

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ABSTRACT Chemokines play a crucial role in developmental processes and recent studies have revealed that they also control gastrulation movements. In this paper, we report the expression patterns of *xSDF-1α*, *xCXCR4* and *xCXCR7* and regulation of the expression of *xSDF-1α* and *xCXCR4* during gastrulation. We performed whole mount *in situ* hybridization (WISH) and quantitative real-time RT-PCR (qRT-PCR) analyses to examine the distribution of transcripts. The effect of activin/nodal signaling on the expression of xSDF-1α and its receptors was examined by animal cap assay and microinjection of *cer-s* mRNA. We have demonstrated that the *xSDF-1α* transcript is increased in the blastocoel roof during gastrulation, but not in the involuted mesoderm. *xCXCR4* was expressed in the mesendoderm at late blastula and was retained throughout gastrulation. *xCXCR7* was found in the dorsal lip around the blastopore in the early gastrula stage and became localized in the presumptive notochord later. We also show that the expression of *xCXCR4* and *xSDF-1α* were reciprocally regulated by activin/nodal signaling. These results suggest that xSDF-1α and its receptors contribute to the cell arrangement of mesoderm cells and their expression patterns are partially regulated by activin/nodal signaling.

KEY WORDS: *SDF-1/CXCL12, CXCR7, chemokine, gastrulation, activin/nodal signaling*

Gastrulation is the process in which cell movements lead to the arrangement of three germ layers in their proper locations. Detailed cell movement during gastrulation in *Xenopus* has been described (Keller and Shook 2004), resulting in better understanding of vertebrate gastrulation. Gastrulation begins with the invagination of cells that form the dorsal lip of future blastopores and later involution creates archenteron. The major driving force of gastrulation seems to be movement of the mesoderm, such as migration of the mesendoderm toward the animal pole and involution of the axial mesoderm, which involves radial and medio-lateral cell intercalation (Keller and Shook 2004).

Chemokines known as chemotactic cytokines are small secreted proteins, produced by a number of hematopoietic and non-hematopoietic stromal cells in adult tissues, that play crucial roles not only in the immune response, but also during various developmental processes (Kucia *et al.,* 2004; Miller *et al.,* 2008; Aman and Piotrowski 2010). One of these chemokines, stromal cell derived factor-1 (SDF-1), also known as CXCL12, functions *via* activation of CXC chemokine receptor 4 (CXCR4), which was first reported as a regulator of lymphocyte chemotaxis. The SDF-1/CXCR4 axis controls the migratory behavior of various types of cells, such as neuronal cell and primordial germ cell migration, stem cell homing, and guidance of lateral line primordial cells (Kucia *et al.,* 2004; Miller *et al.,* 2008), and functions in HIV infection, tumorigenesis, and cancer metastasis (Kucia *et al.,* 2004). SDF-1/CXCR4 signaling also functions in gastrulation (Aman and Piotrowski 2010). In *Xenopus*, the expressions of *xCXCR4* and *xSDF-1α* were found in the mesendoderm and blastocoel roof (BCR), respectively. Furthermore, it was found that SDF-1/CXCR4 signaling was necessary for the migration of mesendoderm cells during gastrulation (Fukui *et al.,* 2007). However, the detailed expression patterns of *xCXCR4* and *xSDF-1α* during gastrulation have remained unclear.

Abbreviations used in this paper: BCR, blastocoel roof; CXCR4, CXC type chemokine receptor 4; CXCR7, CXC type chemokine receptor 7; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; SDF-1, stromal cell-derived factor-1; WISH, whole mount *in situ* hybridization.

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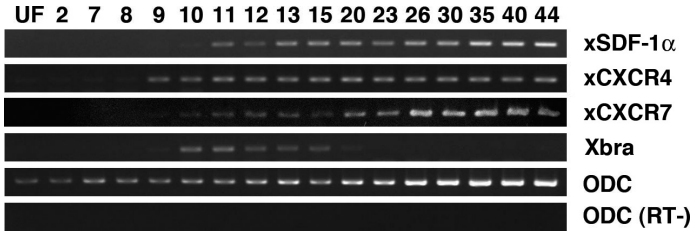


Fig. 1. Expression of *xSDF-1α, xCXCR4* and *xCXCR7*. *RT-PCR analysis of* xSDF-1α, xCXCR4, *and* xCXCR7. *Numbers show the developmental stage of each lane. UF is unfertilized egg.* xCXCR4 *and* xCXCR7 *transcripts increased at the late blastula stage (St. 9) and* xSDF-1α *was detected at the early gastrula stage (St. 10).* Xbra *(*Xenopus brachyury*) was a stage marker and* ODC *(*ornithine decarboxylase*) was a loading control.*

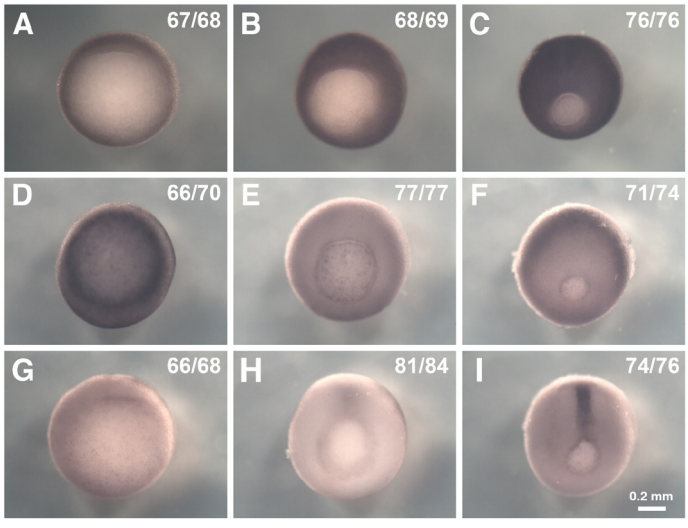
CXCR7, also known as RDC1, binds to SDF-1 and CXCL11 with high affinity (Maksym *et al.,* 2009). Although CXCR7 has been regarded as a decoy receptor due to its inability to activate typical G-protein signaling with the aberration of its G-protein binding domain, recent studies have proposed several mechanisms underlying CXCR7 function. First, CXCR7 scavenges or sequesters SDF-1, consequently generating a concentration gradient of SDF-1 for differential signaling by CXCR4 (Aman and Piotrowski 2010; Maksym *et al.,* 2009). Its second role is to modulate SDF-1-mediated G-protein signaling of CXCR4 by forming the receptor heterodimers regulating SDF-1 chemotaxis in several migrating cells (Levoye *et al.,* 2009). Third, CXCR7 interacts with β-arrestin in a ligand-dependent manner, signals through β-arrestin, and acts as an endogenous β-arrestin-specific receptor (Rajagopal *et al.,* 2010). CXCR7 has a specific role in developing the nervous system and cardiovascular system (Maksym *et al.,* 2009), but its function in earlier development is poorly understood.

In this paper, we describe the expression patterns of *xSDF-1*α, *xCXCR4*, and *xCXCR7* during gastrulation, simultaneously confirmed by whole mount *in situ* hybridization (WISH) and quantitative real-time reverse transcription-PCR (qRT-PCR) analysis. We also examined the regulation of the expression of *xSDF-1*α and *xCXCR4*. These results suggest that SDF-1 signaling supports the migration of the mesendoderm cell cohort toward the animal pole and that activin/nodal signaling acts as a regulator of the expression of *xSDF-1*α and *xCXCR4*, but not *xCXCR7*.

Results

Xenopus SDF-1, CXCR4, and CXCR7 are expressed during gastrulation

To better understand the role and relationship of *xSDF-1*α, *xCXCR4,* and *xCXCR7* during gastrulation, we examined their expression patterns in gastrula stage embryos using RT-PCR, WISH, and qRT-PCR analysis. We previously reported that *xSDF-1*α and *xCXCR4* transcripts increased in early gastrula and late blastula stages, respectively, and remained stable after this stage (Fukui *et al.,* 2007). Here, in addition, the expression of *xCXCR7* was found in the early gastrula stage and also remained stable (Fig. 1). In WISH analysis, *xSDF-1*α transcript was found in the blastocoel roof (BCR) in early gastrula (Fig. 2A) and its expression increased throughout gastrulation (Figs. 2 B,C; 3 A-C). The expression of *xSDF-1*α in each region was confirmed by real-time PCR in separate explants (Fig. 4C). It was observed that *xSDF-1*α was expressed almost equally in animal top, dorsoanimal and dorsal marginal explants but was not expressed in the mesendoderm at the onset of gastrulation. Moreover, increased expression was



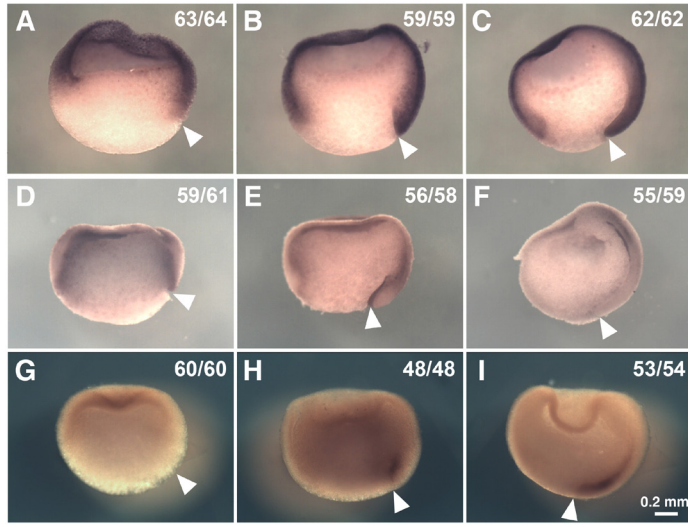


Fig. 2 (left). External view of the expression patterns of *xSDF-1α, xCXCR4* and *xCXCR7*. xSDF-1α, xCXCR4 *and* xCXCR7 *transcripts were examined by WISH in gastrula stage embryos. Panels show the results of probes using* xSDF-1α **(A-C)**, xCXCR4 **(D-F)**, xCXCR7 **(G-I)** *in stage 10* **(A,D,G)**, *stage 11* **(B,E,H)**, *and stage 12* **(C,F,I)** *embryos. Embryos are vegetal view, dorsal side up. Dark blue staining shows signals of probes stained with BM purple. Numbers showing embryos with the same expression pattern to total number of embryos used for the experiment. Scale bar, 0.2 mm.*

Fig. 3 (right). Sagittal view of the expression patterns of *xSDF-1α, xCXCR4* and *xCXCR7*. *WISH was performed in embryos bisected along the midline before probe hybridization. Panels show the results of hybridized probes using* xSDF-1α **(A-C)**, xCXCR4 **(D-F)**, xCXCR7 **(G-I)** *in stage 10* **(A,D,G)**, *stage 11* **(B,E,H)**, *and stage 12* **(C,F,I)** *embryos. Sections are animal pole up and dorsal side to the right. Arrowheads indicate dorsal blastpores. Dark blue staining shows probes stained with BM purple. Numbers show embryos with the same expression pattern to total number of embryos used for the experiment. Scale bar, 0.2 mm.*

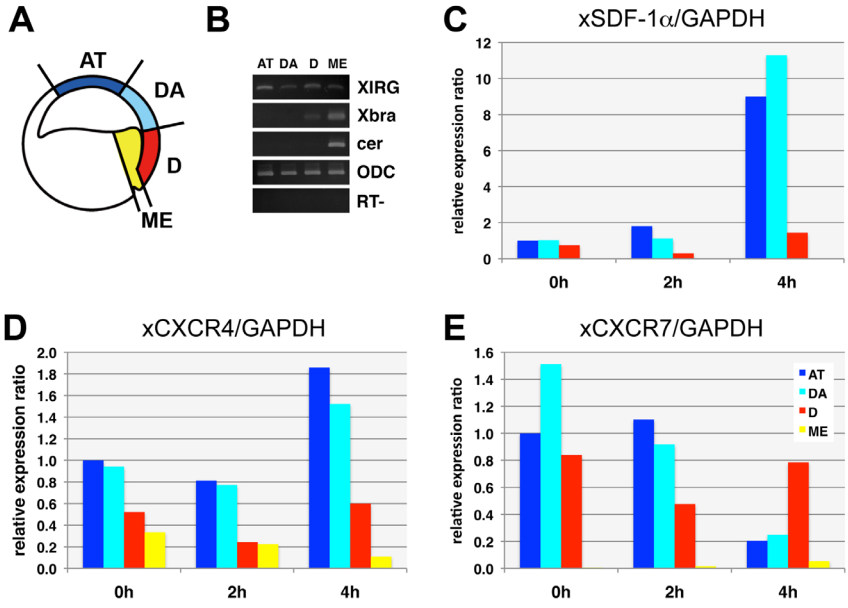
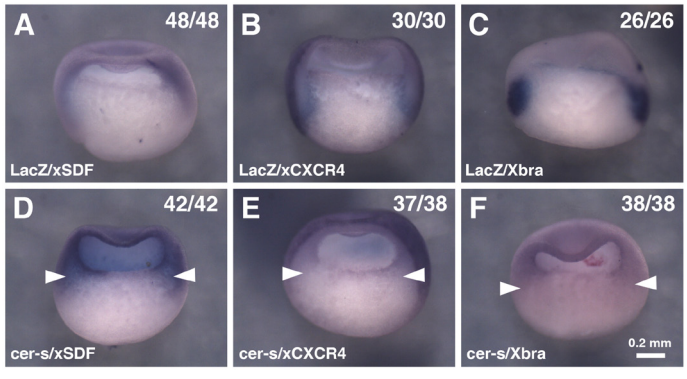


Fig. 4. qRT-PCR analysis of separated explants. (A) *Schematic diagram of the separation. Animal top (AT, blue), dorsoanimal (DA, light blue), dorsal marginal (D, red), mesendoderm (ME, yellow) explants were separated at stage 10 gastrula.* **(B)** *Explant separation was confirmed by RT-PCR using maker genes indicated in the panel as XIRG for BCR, Xbra for pan-mesoderm, and Cer for mesendoderm. ODC was used as a loading control.* **(C,D,E)** *qRT-PCR analysis of* xSDF-1α, xCXCR4, *and* xCXCR7 *expression, respectively. Total RNA was extracted from the explants 0h, 2h, and 4h after separation corresponding to early (st. 10), middle (st. 11), and late (st. 12) gastrula, respectively. Vertical axis shows the ratio of relative expression level normalized by xGAPDH expression to that for AT explant at 0h. Each column in graph is the same color as in (A). Experiment was performed twice and showed a similar tendency.*

observed in animal top and dorsoanimal regions, but not in the dorsal marginal zone throughout gastrulation. These observations suggest that the expression of *xSDF-1α* is localized in non-involuted BCR cells during gastrulation.

xCXCR4 transcription along future blastopores and around the BCR was observed externally at stage 10 (Fig. 2D). It was found that *xCXCR4* was expressed in the dorsal mesendoderm at the onset of gastrulation and expanded towards the ventral side as gastrulation stages advanced. *xCXCR4* expression in the mesoderm was retained throughout gastrulation (Fig. 3 D-F; Fig. S1). In qRT-PCR analysis, *xCXCR4* transcript was detected in mesendodermal, mesodermal, and ectodermal regions. Expression of *xCXCR4* was retained in the mesendoderm and increased in the animal top and dorsoanimal explants (Fig. 4D).



Weak expression of *xCXCR7* was found in the dorsal lip around the blastopore in the early gastrula stage (Fig. 2 G,H). As gastrulation proceeded, *xCXCR7* expression increased in the presumptive notochord (Figs. 2 H,I; 3 H,I). Real-time PCR analysis revealed that *xCXCR7* expression was retained in the dorsal mesoderm explant but decreased in animal top and dorsoanimal explants throughout gastrulation (Fig. 4E). The expression of *xCXCR7* in mesendoderm explants was not detected in early gastrula.

Activin/nodal signaling regulates the expression of xSDF-1α *and* xCXCR4

xCXCR4 was identified as an activin responsive gene in the early gastrula stage (Fukui *et al.*, 2007). Xnrs (*Xenopus* nodal-related proteins) act as mesoderm inducers through activin-like signaling and also play a crucial role in initiating gastrulation (Reissmann *et al.*, 2001); thus, we investigated the role of activin/nodal signals in the regulation of *xSDF-1α*, *xCXCR4*, and *xCXCR7*. A carboxyl-terminal fragment of cerberus, known as cer-s, exhibited potent anti-Xnr activity (Piccolo *et al.*, 1999). Increased *xSDF-1α* and suppressed *xCXCR4* expression in the mesoderm region were observed in *cer-s*-injected embryos by WISH analysis (Fig. 5D, E). Blocking of mesoderm induction by injecting *cer-s* mRNA was confirmed as complete inhibition of *Xbra* expression in the marginal zone (Fig. 5F). These findings suggest that Xnr signaling regulates the expression pattern of *xSDF-1α* and *xCXCR4*.

For further confirmation, we examined the expression of *xSDF-1α* and its receptors in activin-treated animal cap explants. The results obtained by qRT-PCR analysis showed a significant decrease in *xSDF-1α* in explants treated with 5–500 ng/ml activin (Fig. 6A). Furthermore, a significant increase in *xCXCR4* was observed at the concentration of 500 ng/ml activin A (Fig. 6B); however, the expression of *xCXCR7* was unchanged by activin treatment (Fig. 6C). This result suggests that activin signaling regulates the expression of *xCXCR4* and *xSDF-1α* reciprocally, but does not affect that of *xCXCR7*.

Discussion

In this paper, we clarified the expression patterns of *xSDF-1α* and its receptors, *xCXCR4* and *xCXCR7*, during gastrulation. The results of WISH and qRT-PCR are summarized in Fig. 7. Expression of *xSDF-1α* in BCR increased during gastrulation, while no significant gradient was observed in the direction from the animal pole to marginal zone. *xCXCR4* transcript was sustained in mesen-

Fig. 5. Distribution of *xCXCR4* and *xSDF-1α* transcripts in *cer-s* expressing embryos *Upper panels* **(A-C)** *show control LacZ-injected embryos and lower panels* **(D-F)** *are* cer-s-*injected embryos at stage 11.* xSDF-1α **(A,D)**, xCXCR4 **(B,E)**, *and* Xbra **(C,F)** *were used as WISH probes. Dark blue staining shows probes stained with BM purple. Arrowheads show mesendodermal regions. Numbers are embryos with the same expression pattern to total number of embryos used for the experiment. Scale bar, 0.2 mm.*

doderm cells during gastrulation, and relatively weak expression of *xCXCR4* in BCR remained in the anterior-ventral region in the late gastrula stage. Expression of *xCXCR7* was detected in the axial mesoderm, especially dorsally. This is the first observation of the expression pattern of *xCXCR7* during gastrulation.

A time-dependent increase in *xSDF-1α* expression in BCR during gastrulation was observed by WISH and qRT-PCR analyses. Intriguingly, it was also observed that the transcript of *xSDF-1α* did not increase throughout involution of the mesoderm by WISH analysis. This observation was supported by the unchanging *xSDF-1α* expression in the dorsal marginal explant by qRT-PCR analysis (Fig. 4C). Furthermore, the expression of *xSDF-1α* in the animal cap explant was suppressed by activin treatment. These results suggest that *xSDF-1α* transcription in BCR is regulated along with mesodermal differentiation. Since it was reported that a transcription factor *slug* was involved in down-regulation of *SDF-1* (Piva *et al.,* 2011) and the *slug* homologue *snail* was expressed predominantly in the mesoderm in the early gastrula stage (Essex *et al.,* 1993), *slug* homologues could be potential candidates for regulators of *xSDF-1α* transcription in *Xenopus* embryos.

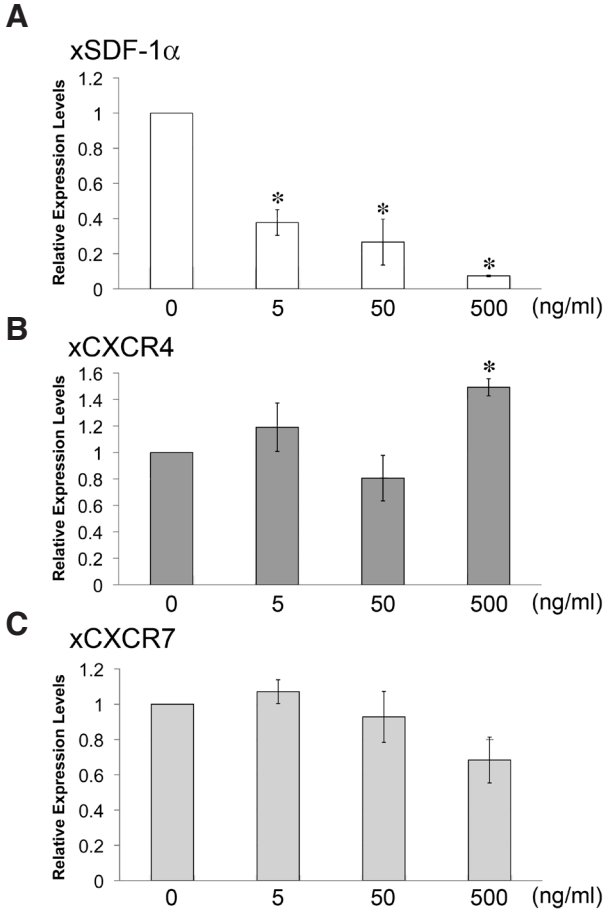


Fig. 6. qRT-PCR analysis of activin-treated explants. *Animal cap explants were treated with 5, 50, and 500 ng/ml activin A at stage 8.5. Expression levels of* xSDF-1α **(A)**, xCXCR4 **(B)**, *and* xCXCR7 **(C)** *were quantified by real-time RT-PCR. Vertical axis shows the ratio of relative expression level normalized by xGAPDH expression to that for the untreated explant. Results are the mean of three independent experiments, and error bars indicate the SE. Differences between means in no treatment (0 ng/ml) and each activin treatment were assessed with Student's t-test. Asterisks (*) indicate p <0.01.*

A previous study demonstrated that a guidance cue for mesoderm cells is contained in the extracellular matrix (ECM) of the inner surface of BCR (Nakatsuji and Johnson 1983). Chemokine SDF-1 binds to fibronectin and heparan sulfate, both of which are components of ECM, which stimulates directional cell migration in hematopoietic cells (Amara *et al.,* 1999; Pelletier *et al.,* 2000). These findings strongly suggest that the xSDF-1 produced in BCR binds to ECM at the inner surface of BCR and acts as a migration cue for mesendoderm cells in the gastrula. It was reported that *Xenopus* PDGF, another candidate for the migration cue, guides the migration of the mesoderm on the stamp of the inner surface of BCR (Nagel *et al.,* 2004). With a similar function and a ligand-receptor expression pattern (Aman and Piotrowski 2010), these two factors may act synergistically or redundantly on mesodermal cells.

Expression of *xCXCR4* in the mesendoderm was suppressed by the expression of *cer-s*, a potent Nodal inhibitor, in the early gastrula stage (Piccolo *et al.,* 1999), and transcript of *xCXCR4* was upregulated by activin, which was revealed by the animal cap assay. These findings suggest that *xCXCR4* is expressed downstream of activin/nodal signaling. Several studies have indicated the molecular mechanism of *xCXCR4* regulation by activin/nodal signaling. *CXCR4* was upregulated by a forkhead transcription factor FoxC in endothelial cells (Hayashi and Kume 2008) and FoxC2 was also regulated by activin in the *Xenopus* gastrula (Pohl and Knöchel 2005), which suggests that FoxC-class transcription factor is a transcriptional regulator for *xCXCR4* under activin/nodal signaling, although expressed in the lateral mesoderm in the gastrula. Furthermore, integrative genomic analyses of *CXCR4* predicted that *CXCR4* was upregulated under activin/nodal signaling and by Sox17 transcription factor (Katoh and Katoh 2010), also one of the activin responsive genes. Taken together, several transcription factors, such as FoxCs and Sox17s, may regulate *xCXCR4* expression under activin/nodal signaling in the *Xenopus* gastrula.

In this experiment, *xCXCR7* expression was observed in the involuting axial mesoderm which followed mesendoderm. The expression pattern of *CXCR7* in the gastrula has been reported only in zebrafish, but was not described well; *zCXCR7b* is expressed in a ring of deep cells in a 6 hpf embryo (Boldajipour *et al*., 2008). Expressions of CXCR4 in leading cells and CXCR7 in trailing cells were also observed in a migrating lateral line primordium (LLP) cell cohort in zebrafish (Aman and Piotrowski 2010). It was proposed that directional cell migration of LLP emerged by the local concentration gradient of SDF-1 due to the sequestration of SDF-1 expressed along the future lateral line by CXCR7 in trailing cells. In the *Xenopus* gastrula, leading xCXCR4-expressing mesendoderm cells migrate animally and xCXCR7-expressing involuting axial mesodermal cells follow them on xSDF-1α-expressing BCR cells (Fig. 7). It is considered that formation of a local gradient is also induced by the incorporation of SDF-1 produced ubiquitously in BCR into xCXCR7-expressing mesoderm cells.

In conclusion, we have shown here the expression patterns of *xSDF-1α* and its receptors. xCXCR4-expressing mesendoderm cells are followed by xCXCR7-expressing involuting mesoderm cells and BCR cells, which become a substrate for migrating mesoderm expressing xSDF-1α during *Xenopus* gastrulation. These findings suggest that SDF-1 and CXCR4 contribute to the migration of mesoderm cells and our results will shed light on the

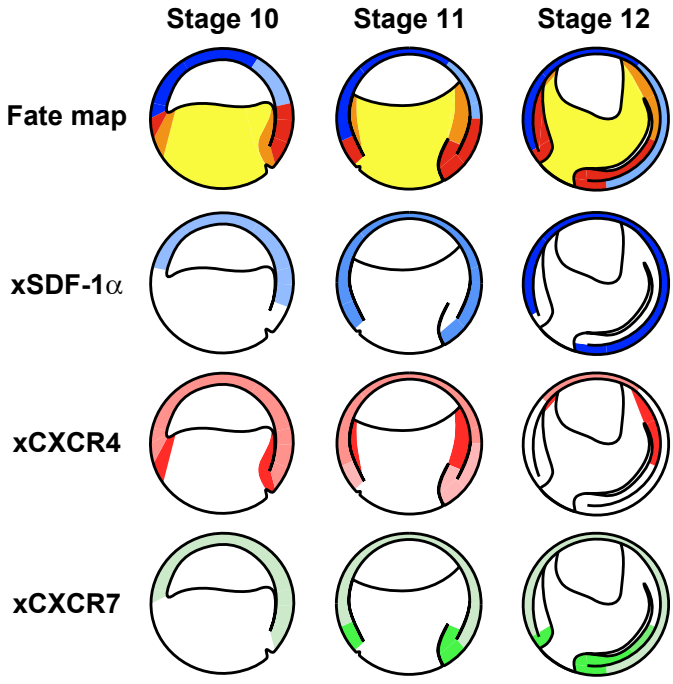


Fig. 7. Schematic diagram of the fate map of *Xenopus* gastrula and expression patterns of *xSDF-1α*, *xCXCR4* and *xCXCR7*. *Diagrams in top row show the fate map of sagittal section of gastrula embryo stages from 10 to 12 and colors in diagrams indicate mesendoderm (orange), involuting mesoderm (red), presumptive neural tissue (light blue), and future epidermis (blue). Diagrams below are expression patterns of* xSDF-1α *(blue),* xCXCR4 *(red), and* xCXCR7 *(green) in stages corresponding with the fate map, respectively. Concentration of each color in the expression patterns corresponds to the predicted amount of expression of each mRNA examined by WISH and qRT-PCR analyses.*

role of CXCR7 in orchestrated cell migration during gastrulation.

Materials and Methods

Preparation of embryos

Xenopus embryos were obtained from adult females by injecting them with human chorionic gonadotropin (ASKA, Japan) at a dose of 300 IU. After artificial fertilization, the embryos were maintained in Steinberg's solution (58.2 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 4.6 mM Tris–HCl, pH 7.4), and dejellied with 4.5% cysteine hydrochloride in Steinberg's solution (pH 8.0). Developmental stages are according to Nieukoop and Faber.

RT-PCR and whole-mount in situ *hybridization*

Primers and digoxigenin-labeled antisense RNA probes for *xSDF-1α* and *xCXCR4* were described previously (Fukui *et al.*, 2007). *xCXCR7* primers were forward 5'- TGCTCCACTGCTGTATCAACCC-3' and reverse 5'- AGGAATGTAAGCCACTTTGGTCC-3'. The *xCXCR7* probe was prepared from NIBB Mochii normalized *Xenopus* tailbud library clone number Xl065g11 (gene accession number BJ060310). Whole-mount *in situ* hybridization was performed by the partially modified method of Harland. Briefly, embryos were fixed with MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde, pH 7.4) and boiled in absolute ethanol for 3 minutes instead of proteinase K treatment. The probes were hybridized for 48 hours at 58°C. BM purple was used as the color reagent.

Microsurgery

Animal top, dorsoanimal, dorsal marginal, and mesendoderm explants were separated from embryos at stage 10 and cultured in Steinberg's solution (Fig. 4A). Animal caps (i.e., presumptive ectoderm explants) were dissected at stage 8.5 in Steinberg's solution, treated with the appropriate concentration of activin A for 1 hour, washed twice, and cultured for 4 hours at room temperature for total RNA isolation.

Microinjection

Constructs in pCS2 vector were cut by Not I and capped mRNAs were prepared using the mMASSAGE mMACHINE SP6 kit according to the instruction manual (Ambion). For WISH, 2 ng mRNA encoding cer-s (a kind gift from Dr. H. Kuroda, Shizuoka Univ.) was injected into the vegetal side of four blastomeres of a 4-cell stage embryo.

Quantitative real-time reverse transcription-PCR (qRT-PCR) analysis

Total RNA was extracted from 3 (separation) or 5 (animal cap assay) explants by ISOGEN (NipponGene, Japan) or TriPureIsolation Reagent (Roche) according to the instruction manuals. Random primed reverse-transcription was performed using total RNAs as a template. Quantitative real-time PCR was performed on an ABI PRISM 7700 (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the instruction manual. Real-time-PCR assays were performed using the following primers: forward 5'-CAGAACATTATTCCCGCCTCAAC-3' and reverse 5'- AACTTTTCCGACAGCCTTTGC-3' for xGAPDH (BC043972); forward 5'-TGTGACGGCTAACCTGGGAATG-3' and reverse 5'- CCAATACCAATCGTTGAGTGTCTCC-3' for *xSDF-1α* (BC073527); forward 5'- TGCGTGTGTCTTGAAAGTAGG-3' and reverse 5'-CACTGGGATGATTTATGAATCTG-3' for *xCXCR4* (BC073603); and forward 5'- ATCTGAATGGGGCAACTGGG -3' and reverse 5'-ATCTGAATGGGGCAACTGGG-3' for xCXCR7 (BC098974). Primers for *xSDF-1α*, *xCXCR4*, and *xCXCR7* were designed in 3'-UTR to detect endogenous transcripts.

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