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Characterization of CXC-type chemokine molecules in early *Xenopus laevis* development

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ABSTRACT Chemokine molecules play important roles in the immune system. However, several chemokine molecules are expressed during early development before the immune system is established. Using reverse transcription–polymerase chain reaction (RT-PCR) and overexpression of chemokine molecules, we identified and characterized *Xenopus laevis* CXC-type chemokine ligands (*XCXCL13L1, XCXCL13L2, XCXCLa, XCXCLb, XCXCLd,* and *XCXCLe*) and receptors (*XCXCR1/2, XCXCR3, XCXCR5, XCXCR6,* and *XCXCRa*) during early development. The CXC-type ligands have low identity with genes for human CXC ligands (CXCL). With the exception of *XCXCRa*, the CXC receptors (CXCR) identified in the present study had high (~40%–65%) identity with human CXCR genes. Although the expression patterns for the *CXCL* and *CXCR* genes differed, transcript levels for all genes were very low during early embryogenesis. Overexpression of *XCXCL13L1, XCXCL13L2, XCXCLa, XCXCR3, XCXCR6,* and *XCXCRa* interfered with gastrulation and neural fold closure. The results of the present study suggest that several chemokine molecules are related to cell movements during early morphogenesis.

KEY WORDS: *chemokine, CXC receptor, CXC ligand, gastrulation, Xenopus laevis*

Introduction

Chemokine ligands are considered cytokine molecules and, in mammals, have been investigated primarily in terms of their immunomodulatory role. Mammalian chemokines are divided into four families, namely CC-, CXC-, XC-, and CX3C-type chemokines, which contain 28, 17, 2, and 1 member, respectively (Laing and Secombes, 2004; Hiraoka *et al.*, 2011). Chemokine receptors are members of the G-protein-coupled receptor (GPCR) family and have seven transmembrane structures. The chemokine receptors are also divided into four families according to ligand type: CC-, CXC-, XC-, and CX3C-type receptors, containing 10, 7, 1, and 1 member, respectively (Allen *et al.*, 2007).

Seventeen CXC-type chemokines have been identified and characterized in mammals (Laing and Secombes, 2004). The CXC chemokines are further divided into two groups depending on the presence (+) or absence (–) of the tripeptide motif glutamic acid–leucine–arginine (ELR) at the N-terminus of the first cysteine residue. The CXC ligands (CXCL) CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, and CXCL15 belong to the ELR(+) group (Romagnani *et al.*, 2004; Wang *et al.*, 2005). It has been reported that CXCL1–CXCL8 bind to the CXC receptor (CXCR) CXCR2, but a receptor for CXCL15 is yet to be unidentified (Zlotnik and Yoshie, 2000; Wang *et al.*, 2005). Specifically, CXCR1 binds CXCL1, CXCL6, and CXCL8 (Zlotnik and Yoshie, 2000); CXCR3 and its alternatively splicing variant bind CXCL4, CXCL9, CXCL10, and CXCL11 (Zlotnik and Yoshie, 2000; Lasagni *et al.*, 2003); CXCR4 binds CXCL12; CXCR5 binds CXCL13; CXCR6 binds CXCL16; and, CXCR7 binds CXCL11 and CXCL12 (Zlotnik and Yoshie, 2000; Naumann *et al.*, 2010; Agostini *et al.*, 2005). No receptors have been identified as yet for CXCL14 and CXCL17 (Hara and Tanegashima, 2012; Hiraoka *et al.*, 2011).

Recent studies have revealed that several chemokine ligands and receptors have important roles in early embryogenesis in vertebrates before the immune system is established. For example, CXCL12/CXCR4 promotes directional movement of primordial germ cell (PGC) migration in zebrafish (Doitsidou *et al.*,

Abbreviations used in this paper: CXCL, CXC ligand; CXCR, CXC receptor; PGC, primordial germ cell; RT-PCR, reverse transcription-polymerase chain reaction.

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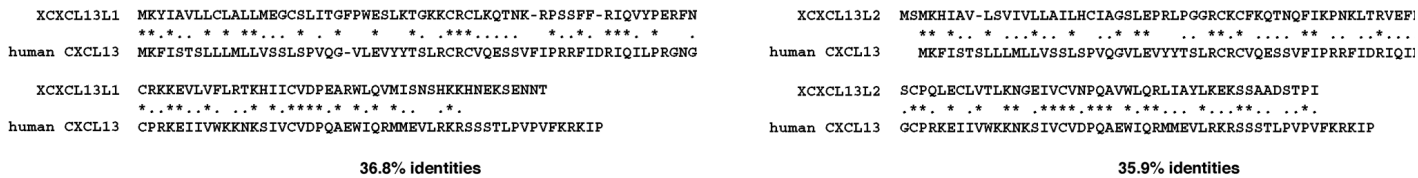
A



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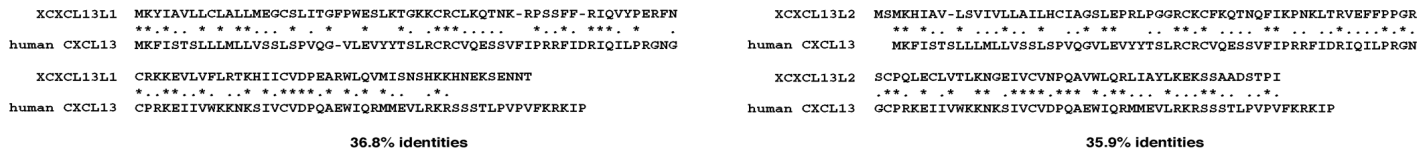
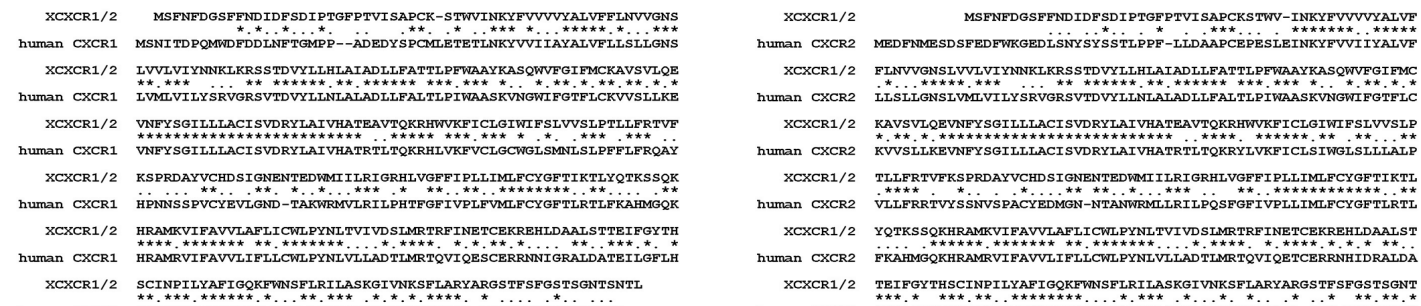
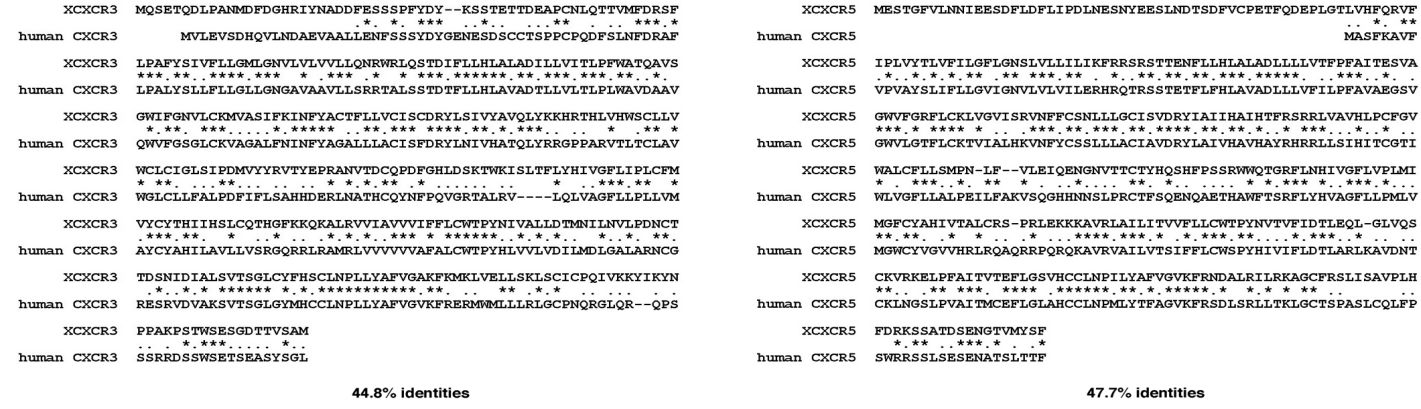






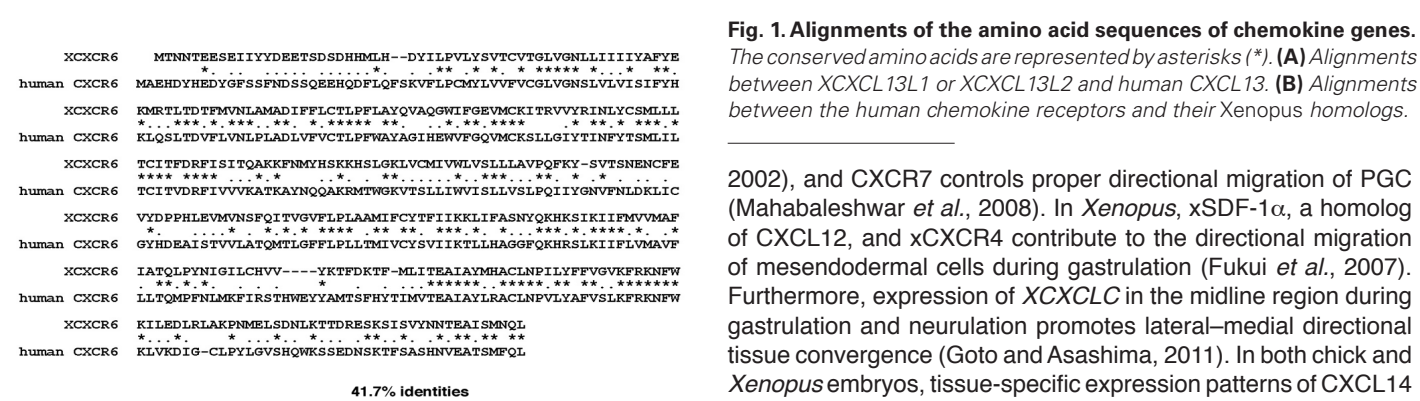


Fig. 1. Alignments of the amino acid sequences of chemokine genes. *The conserved amino acids are represented by asterisks (*).* **(A)** *Alignments between XCXCL13L1 or XCXCL13L2 and human CXCL13.* **(B)** *Alignments between the human chemokine receptors and their* Xenopus *homologs.*

2002), and CXCR7 controls proper directional migration of PGC (Mahabaleshwar *et al.*, 2008). In *Xenopus*, xSDF-1α, a homolog of CXCL12, and xCXCR4 contribute to the directional migration of mesendodermal cells during gastrulation (Fukui *et al.*, 2007). Furthermore, expression of *XCXCLC* in the midline region during gastrulation and neurulation promotes lateral–medial directional tissue convergence (Goto and Asashima, 2011). In both chick and *Xenopus* embryos, tissue-specific expression patterns of CXCL14

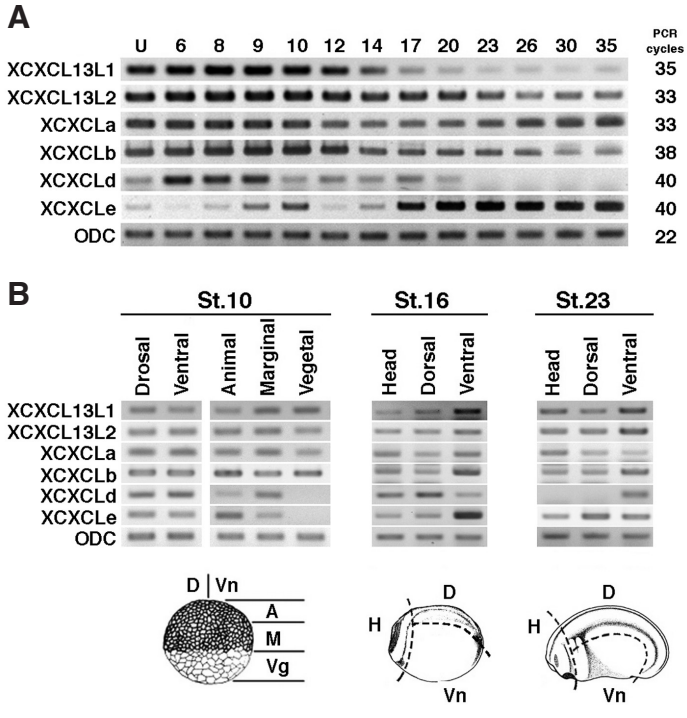
have been reported during early embryogenesis. However, many chemokine ligands and receptors remain uncharacterized in embryogenesis.

In the present study, we cloned the following CXC-type chemokine ligands from *Xenopus laevis*: *CXCL13L1* (*XCXCR13L1*: GenBank/DDBJ Accession no. BG018851); *CXCL13L2* (*XCXCL13L2*: CF342082); *CXCLa* (*XCXCLa*: CB199271); *CXCLb* (*XCXCLb*: BC130085); *CXCLd* (*XCXCLd*: AW644053); and *CXCLe* (*XCXCLe*: CF548813). In addition, we cloned the *CXCR1/2* (*XCXCR1/2*: AJ312936) receptor and identified four novel chemokine receptors: *CXCR3* (*XCXCR3*: AB720054); *CXCR5* (*XCXCR5*: AB720055); *CXCR6* (*XCXCR6*: AB720056); and *CXCRa* (*XCXCRa*: AB720057). The nomenclature of these chemokine genes is based on the Cytokine Family Database (http://cmbi.bjmu.edu.cn/cmbidata/cgf/CGF_Database/cytokine.medic.kumamoto-u.ac.jp/default.htm). The expression patterns of these chemokine genes and their effects on early embryogenesis were evaluated.

Results

Isolation of Xenopus laevis CXCL *and* CXCR *genes*

Full-length cDNA fragments were obtained from Stage 10 embryos for *XCXCR13L1*, *XCXCL13L2*, *XCXCLa*, *XCXCLb*, *XCXCLd*, *XCXCLe*, *XCXCR1/2*, *XCXCR3*, *XCXCR5*, *XCXCR6*, and *XCXCRa* using reverse transcription–polymerase chain reaction (RT-PCR) (see Materials and Methods). The nucleotide sequence of *XCXCR1/2* is same as one of CXCR2 like protein (AJ312936). The amino acid sequence of XCXCR1/2 is also same as one of *Xenopus laevis* chemokine (C-X-C motif) receptor 1 (cxcr1) (NCBI NM_001088765.1) that has 2-base-pair mismatches. Therefore we named this gene XCXCR1/2. XCXCLa and XCXCLe contain the ELR motif at the N-terminus of the first cysteine residue, but do not exhibit high identity with mammalian chemokine molecules containing the ELR motif in their amino acid sequence. XCXCLb and XCXCLd do not contain the ELR motif and have low identity with mammalian CXCLs. XCXCL13L1 and XCXCL13L2 do not contain the ELR motif either, but they have slightly higher identity (36.8% and 35.9%, respectively) with human CXCL13 (NCBI NP_006410.1) (Fig. 1A). The identity between XCXCL13L1 and XCXCL13L2 is not high (37.9%). The predicted XCXCR proteins contain the seven transmembrane regions that are conserved in GPCR. XCXCR1/2 exhibits 55.8% and 58.3% identity with human CXCR1 (NCBI NP_000625.1) and human CXCR2 (NCBI NP_001548.1), respectively; human CXCR1 have a high identity (79.0%) with human CXCR2. We were not able to find any *Xenopus* CXCR1- or CXCR2-like genes in the gene databank. These observations suggest that *XCXCR1/2* is an ancestor gene of both *CXCR1* and *CXCR2*. XCXCR3, XCXCR5, and XCXCR6 have high



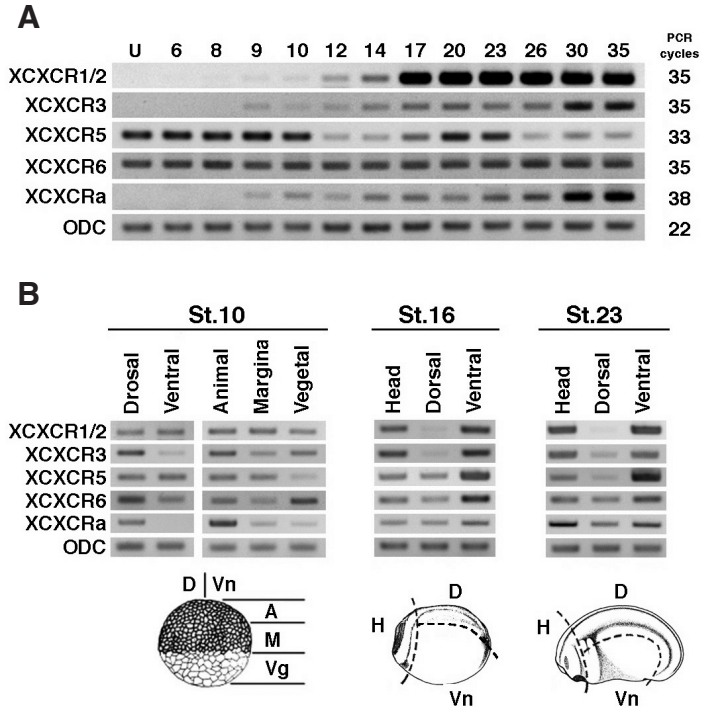


Fig. 2 (left). Expression patterns of *Xenopus* chemokine ligand transcripts. *Reverse transcription–polymerase chain reaction analysis was performed using total RNA extracted from* Xenopus *embryos at different stages of development and from different regions.* Ornithine decarboxylase *(*ODC*) was used as an internal control.* **(A)** *Temporal expression patterns. U, unfertilized eggs. The numbers indicate developmental stages. The number of polymerase chain reaction (PCR) cycles is given on the right.* **(B)** *Spatial expression patterns. Embryos were dissected at the stages indicated, and dissections were performed as shown in the bottom panels. Abbreviations: D, dorsal; Vn, ventral; A, animal; M, marginal; Vg, vegetal; H, head.*

Fig. 3 (right). Expression patterns of *Xenopus* chemokine receptor transcripts. *Reverse transcription–polymerase chain reaction analysis was performed using total RNA extracted from* Xenopus *embryos at different stages of development and from different regions.* Ornithine decarboxylase *(*ODC*) was used as an internal control.* **(A)** *Temporal expression patterns. U, unfertilized eggs. The numbers indicate developmental stages. The number of polymerase chain reaction (PCR) cycles is given on the right.* **(B)** *Spatial expression patterns. Embryos were dissected at the stages indicated, and dissections were performed as shown in the bottom panels. Abbreviations as in Fig. 2.*

identities of 44.8%, 47.7%, and 41.7% with human CXCR3 (NCBI NP_001495.1), CXCR5 (NCBI NP_001707.1), and CXCR6 (NCBI NP_006555.1), respectively (Fig. 1B). XCXCRa does not exhibit high identity with any mammalian CXCR.

Expression of Xenopus CXCL *genes during morphogenesis*

Because only low levels of *Xenopus* CXC-type chemokine ligand transcripts were present during early embryogenesis, we could not determine their localization using whole-mount *in situ* hybridization. Therefore, we investigated the expression patterns of these genes using RT-PCR analysis. Transcripts of *XCXCL13L1*, *XCXCL13L2*, *XCXCLb*, and *XCXCLd* are expressed maternally, with levels decreasing after the gastrula stage. Maternal expression of *XCXCLa* decreases gradually during the gastrula and neurula stages, and increases again after the tailbud stage. *XCXCLe* expression has a faint peak at the early gastrula stage and increases after the late neurula stage (Fig. 2A). At the gastrula stage, there is uniform distribution of *XCXCL13L1*, *XCXCL13L2*, *XCXCLa*, and *XCXCLb* throughout the embryo, whereas the expression of *XCXCLd* and *XCXCLe* increases in the marginal and animal regions, respectively (Fig. 2B, left panel). Transcripts of all the *XCXCL* genes except *XCXCLa* are abundant in the ventral region at the later stages of embryogenesis (Fig. 2B, center and right panels).

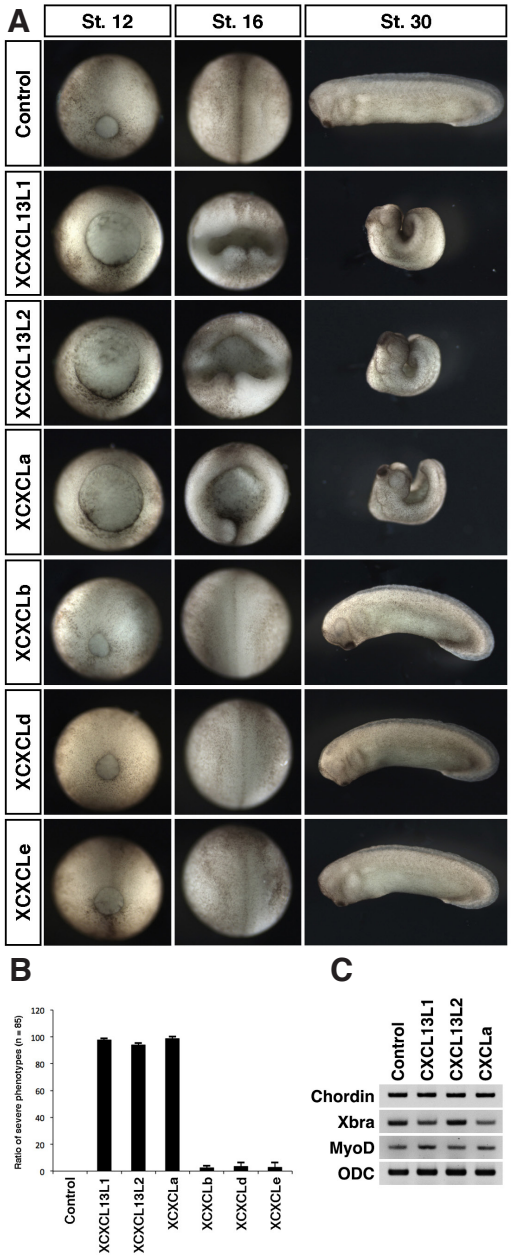


Fig. 4. Phenotypes of *Xenopus* embryos injected with chemokine ligands into the dorsal side. (A) *Phenotypes of the injected embryos. The mRNA of each chemokine ligand (500 pg/blastomere) was injected into two dorsal blastomeres of 4-cell-stage embryos. Left panels, Stage 12, vegetal view; center panels, Stage 16, dorsal view; right panels, Stage 30, lateral view.* **(B)** *The ratio of the injected embryos exhibiting gastrulation defects.* **(C)** *RT-PCR analysis of mesodermal marker genes. Total RNA for RT-PCR was extracted the dorsal sectors of the injected embryos at stage 10.*

Expression of Xenopus CXCR *genes during early development*

XCXCR1/2, *XCXCR3*, and *XCXCRa* are expressed zygotically and their expressions increase gradually. Maternal expression of *XCXCR5* decreases during the late gastrula and early neurula stages, and increases after the late neurula stage. Expression of *XCXCR6* is uniform throughout the early embryonic stages (Fig. 3A). As for CXCL, transcript levels of *Xenopus* CXC-type chemokine receptors are too low during early embryogenesis to enable their specific localization by whole-mount *in situ* hybridization. Thus, RT-PCR analysis was performed using region-specific RNA sources. *XCXCR1/2* transcripts were distributed uniformly at the gastrula stage, whereas *XCXCR3* and *XCXCRa* are abundantly expressed in the dorsal animal region. Abundant expression of the *XCXCR5* transcript is found in the animal and marginal regions, whereas *XCXCR6* is abundant in the dorsal vegetal region (Fig. 3B, left panel). During the later stages of embryogenesis, the transcripts of all *XCXCR* genes are expressed mainly in the head and ventral regions (Fig. 3B, center and right panels).

Overexpression of XCXCL13L1, XCXCL13L2*, and* XCXCLa *interfered with gastrulation*

Dorsal overexpression of *xSDF1-alpha* (*CXCL12*) and *XCXCLC* inhibited gastrulation and neurulation in *Xenopus* (Fukui *et al.*, 2007; Goto and Asashima, 2011). To investigate whether the *XCXCL* genes affect early morphogenesis, we injected each synthetic mRNA encoding the full-length *XCXCL* gene into two dorsal blastomeres of the 4-cell embryo. The injected embryos developed normally until the formation of the blastopore (data not shown). Injection of *XCXCLb*, *XCXCLd*, and *XCXCLe* mRNA had no effect on embryogenesis at later stages (Fig. 4A). However, overexpression of *XCXCL13L1, XCXCL13L2*, and *XCXCLa* delayed dorsal lip closure in the injected embryos. In addition, the neural folds failed to close during neurulation and these embryos had very short anterior–posterior axes (Fig. 4A). Most of *XCXCL13L1-, XCXCL13L2-* and *XCXCLa*-injected embryos showed severely gastrulation-inhibited phenotypes (Fig. 4B). When we investigated expressions of mesodermal maker genes, *Chordin*, *Xbra* and *MyoD* in the *XCXCL13L1-, XCXCL13L2-* and *XCXCLa*-injected embryos, *XCXCL13L2* did not affect expression of mesodermal marker genes (Fig. 4C). However, expressions of *Xbra* were reduced in the *XCXCL13L1-* and *XCXCLa*-injected embryos (Fig. 4C). And we confirmed ventral overexpression of *XCXCL13L1, XCXCL13L2* or *XCXCLa* mRNA did not affect ventral morphogenesis (Fig. 6A-D,H).

Overexpression of XCXCR3, XCXCR6, *and* XCXCRa *interfered with gastrulation*

Dorsal overexpression of *xCXCR4* inhibited gastrulation and neurulation in *Xenopus* (Fukui *et al.*, 2007), and CXCL12b/CXCR4a signaling is necessary for zebrafish morphogenesis (Nair and Schilling, 2008). To test whether *XCXCR* genes have any effect on *Xenopus* morphogenesis, each synthetic mRNA encoding the full-length *XCXCR* gene was injected into two dorsal blastomeres of the 4-cell embryo. The injected embryos developed normally until the formation of the blastopore (data not shown). Injection of *XCXCR3*, *XCXCR6*, and *XCXCRa* mRNA interfered with gastrulation and neurulation (Fig. 5A). These embryos also had very short anterior–posterior axes and widely opened neural tubes (Fig. 5A), similar to that seen following injection of *XCXCL13L1, XCXCL13L2*, and *XCXCLa* (Fig. 4A). Severely gastrulation-inhibited phenotypes were shown by a high incidence in the *XCXCR3*-, *XCXCR6*-, and *XCXCR*-injected embryos (Fig. 5B). Expressions of mesodermal maker genes were unchanged in the *XCXCR3*-, *XCXCR6*-, and *XCXCR*-injected embryos (Fig. 5C). When we injected *XCXCR3*, *XCXCR6* or *XCXCRa* mRNA into two ventral blastomeres of the 4-cell embryo, *XCXCR6* interfered with the invagination of ventral side (Fig. 6 A,F,H). However ventral injection of *XCXCR3* and *XCXCRa* did not affect ventral morphogenesis (Fig. 6 E,G,H).

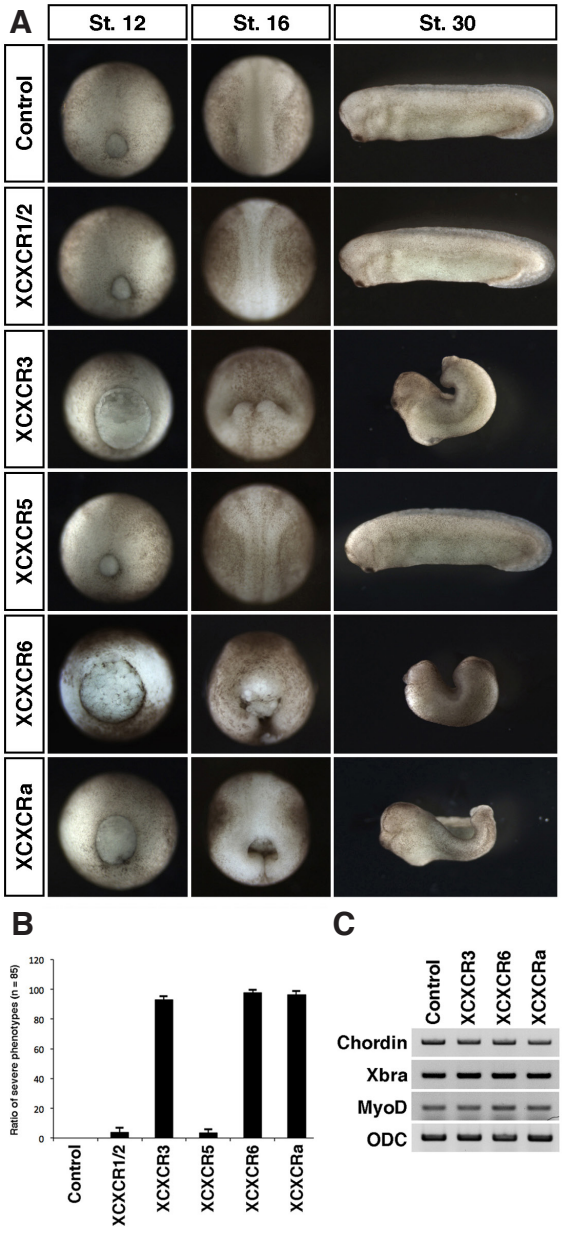


Fig. 5. Phenotypes of *Xenopus* embryos injected with chemokine receptors into the dorsal side. (A) *Phenotypes of the injected embryos. The mRNA of each chemokine receptor (500 pg/blastomere) was injected into two dorsal blastomeres of 4-cell-stage embryos. Left panels, Stage 12, vegetal view; center panels, Stage 16, dorsal view; right panels, Stage 30, lateral view.* **(B)** *The ratio of the injected embryos exhibiting gastrulation defects.* **(C)** *RT-PCR analysis of mesodermal marker genes. Total RNA for RT-PCR was extracted the dorsal sectors of the injected embryos at stage 10.*

Discussion

In mammals, each chemokine receptor binds with one or more chemokine ligands (Zlotnik and Yoshie, 2000; Lasagni *et al.*, 2003; Wang *et al.*, 2005; Agostini *et al.*, 2005; Naumann *et al.*, 2010). With the exception of XCXCRa, *Xenopus* CXC-type chemokine receptors exhibit high identity with mammalian receptors. Specifically, the amino acid sequences of *Xenopus* CXCL12 (SDF1) and CXCL14 exhibit high identity with their mammalian homologues, and their expression patterns are partly conserved in vertebrates (Fukui *et al.*, 2007; Park *et al.*, 2009). The interaction between CXCL12 (SDF1) and CXCR4 is particularly conserved in vertebrates. On this basis, we considered that *Xenopus* CXC-type chemokine ligands would exhibit high identity with homologous mammalian ligands that interact with mammalian chemokine receptors. However, the amino acid sequences of *Xenopus* CXC-type chemokine ligands that were characterized in the present study exhibit only low identity with the mammalian ligands, and we could not find *Xenopus* cDNA sequences exhibiting high identity with mammalian chemokine ligands in the gene databank. These findings suggest that most of the *Xenopus* CXC-type chemokine ligands may have evolved specifically.

Dorsal cell movements are important for early morphogenesis (Keller and Tibbetts, 1989; Winklbauer, 1990), and chemokine molecules play important roles in cell migration during morphogenesis (Doitsidou *et al.*, 2002; Fukui *et al.*, 2007; Nair and Schilling, 2008; Goto and Asashima, 2011). It has been previously reported that *Xenopus* CXCR4 is expressed in the dorsal mesendodermal region and that CXCL12 (SDF1)/CXCR4 signaling mediates the directional movement of mesendodermal cells during *Xenopus* gastrulation (Fukui *et al.*, 2007). In the present study, we demonstrated that dorsal overexpression of *XCXCR3* and *XCXCRa* interfered with gastrulation (Fig. 5), and that the *XCXCR3* and *XCXCRa* transcripts are expressed dorsally during the gastrula stage (Fig. 3). Moreover, ventral overexpression of *XCXCR3* and *XCXCRa* did not affect morphogenesis (Fig. 6). These suggest that *XCXCR3* and *XCXCRa* might play main roles in dorsal morphogenesis. On the other hand, overexpression of *XCXCR6* interfered with morphogenesis at the dorsal and ventral side (Fig. 5, 6), and expression level of *XCXCR6* at the ventral side is higher than those of *XCXCR3* and *XCXCRa* at the gastrula stage (Fig. 3). These results suggest that *XCXCR6* might function on both dorsal and ventral side.

Overexpression of *XCXCL13L1*, *XCXCL13L2*, and *XCXCLa* also interfered with gastrulation (Fig. 4), but the transcripts of these genes

are uniformly expressed at the gastrula stage. *Xenopus* CXCL12 (SDF1), which attracts CXCR4-expressing mesendodermal cells during gastrulation, is also expressed uniformly in the ectodermal region during the gastrula stage (Fukui *et al.*, 2007). Conversely, *XCXCLC* expression in the midline region during gastrulation affects lateral–medial directional tissue convergence (Goto and Asashima, 2011). Thus, there are variations in the expression patterns of transcripts for *Xenopus* chemokine ligands that affect cell movements, rather than in the expression of chemokine receptors. Moreover, ventral injection of *XCXCL13L1*, *XCXCL13L2*, and *XCXCLa* did not affect morphogenesis (Fig. 6). We suggest that the protein distribution of the secreted chemokine ligands or the pattern of expression of their receptors is more important than the expression patterns of ligand transcripts.

Overexpression of *XCXCL13L2*, *XCXCR3*, *XCXCR6* and *XCXCRa* interfered with gastrulation but did not affect expressions of mesodermal marker genes. We suggest that these four chemokine molecules might contribute to only cell movements during early morphogenesis. On the other hand, overexpression of *XCXCL13L1* and *XCXCLa* interfered with morphogenesis and reduced *Xbra* expression in the injected embryos. These suggest that cell differentiation affected by *XCXCL13L1* and *XCXCLa* would cause defects of morphogenesis in part.

In mammals, CXCR1 and CXCR2 interact with ELR(+) CXC-type chemokine ligands (Romagnani *et al.*, 2004). In the present study, only XCXCLa has the ELR motif and its overexpression inhibited gastrulation (Fig. 5). However, overexpression of *XCXCR1/2* had no effect on morphogenesis (Fig. 5). We suggest that the candidate receptor for *XCXCLa* is not *XCXCR1/2*, but may be another CXC-type receptor, including XCXCR4 or XCXCR7. Human CXCL13 interacts with CXCR5 (Legler *et al.*, 1998). Both XCXCL13L1 and XCXCL13L2 have slightly higher identities with human CXCL13 (Fig. 1A), and their overexpression affected early morphogenesis in *Xenopus* (Fig. 4). However, *XCXCR5* had no effect on morphogenesis (Fig. 5). These suggest that *XCXCR5* is not a candidate chemokine receptor for *XCXCL13L1* and *XCXCL13L2*. CXCR3 interacts with several ELR(-)–CXC-type chemokine ligands, such as CXCL4, CXCL9, CXCL10, and CXCL11 (Romagnani *et al.*, 2004), and CXCR6 selectively binds CXCL16 in mammals (Agostini *et al.*, 2005). However, we could not find any homologous *Xenopus* ligands for CXCR3 and CXCR6 in the gene databank. Regardless of the differences in amino acid sequences between mammalian and *Xenopus* CXCL, the *Xenopus* CXC-type chemokine ligands may have functional redundancies with mammalian ligands.

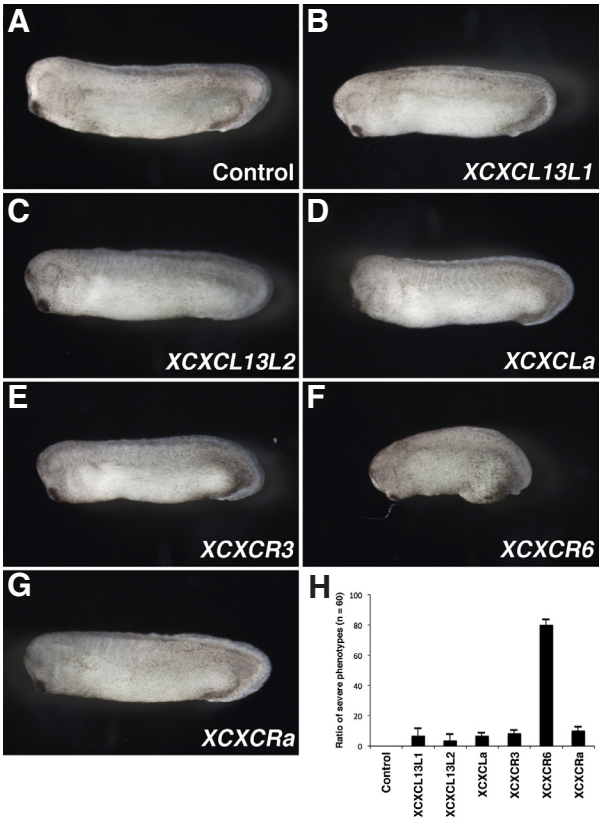


Fig. 6. Phenotypes of *Xenopus* embryos injected with chemokine receptors into the dorsal side. (A-G) *Phenotypes of the injected embryos.* **(A)** *Control.* **(B)** XCXCL13L1. **(C)** XCXCL13L2. **(D)** XCXCLa. **(E)** XCXCR3. **(F)** XCXCR6. **(G)** XCXCRa. **(H)** *The ratio of the injected embryos exhibiting ventral morphogenesis defects.*

TABLE 1

PRIMER PAIRS USED TO GENERATE FULL-LENGTH cDNA OF EACH GENE FOR CLONING

Gene	Primer
XCXCL13L1	Forward: 5′-CCCCGAATTCTCAAGGTACAATGAAGTACA-3′ Reverse: 5′-CCCCCTCGAGTTAAGTATTATTTTCACTTT-3′
XCXCL13L2	Forward: 5′-CCCCATCGATCAGATCCAGTATGAGCATGA-3′ Reverse: 5′-CCCTCTCGAGTCATATTGGTGTACTGTCTG-3′
XCXCLa	Forward: 5′-ACCCATCGATAATCTCCAACATGCAGTGCC-3′ Reverse: 5′-CCCCCTCGAGTCATTTCTTCTGTGAATTCC-3′
XCXCLb	Forward: 5′-CCCCGAATTCAACAGCCAAAATGAGTGCTA-3′ Reverse: 5′-CCAACTCGAGTTAGCGATCTGTTTGAGTCA-3′
XCXCLd	Forward: 5′-CCCCGAATTCACTGAAAGCTATGACACTGT-3′ Reverse: 5′-CCTTCTCGAGTGCCAAAGCATCGAGAGATA-3′
XCXCLe	Forward: 5′-CCCCGAATTCCCAATTTACCATGGAAACCA-3′ Reverse: 5′-CCCTCTCGAGTTAAAGAGCAGAAACAGGTG-3′
XCXCR1/2	Forward: 5′-CCCCATCGATATGTCCTTTAATTTTGATGG-3′ Reverse: 5′-CCCCGAATTCTTAGAGTGTGTTGGATGTGT-3′
XCXCR3	Forward: 5′-CCCCGAATTCACTTCTACAAATGCAAAGTG-3′ Reverse: 5′-TCCCCTCGAGTCACATTGCAGATACTGTAG-3′
XCXCR5	Forward: 5′-GAAAGAATTCGAGTGGAGGAATGGAGTCTA-3′ Reverse: 5′-GGGGCTCGAGTCATCATTAAAAAGGCACAC-3′
XCXCR6	Forward: 5′-CCGAATTCCGTAAATAATATGACAAACAACACAGAAGA-3′ Reverse: 5′-GGGGCTCGAGTTACAACTGGTTCATACTAA-3′
XCXCRa	Forward: 5′-GGGGGAATTCTGTGAGCAATATGGCAGAAG-3′ Reverse: 5′-TTTGCTCGAGCAGCAAATGTGAAATAGACC-3′

Materials & Methods

Cloning of Xenopus *CXC-type chemokine ligands and receptors*

The cDNA of each gene was generated by RT-PCR of RNA samples from Stage 10 embryos. Total RNA was prepared using TRIzol (Invitrogen). cDNA synthesis was carried out using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Table 1 lists the primer pairs used for RT-PCR cloning of chemokine ligands and receptors. PCR was performed using KOD-Plus DNA polymerase (TOYOBO). In order to obtain the full-length cDNA of chemokine ligands and receptors, the primers were designed with reference to the sequences of BG018851 for *XCXCL13L1*, CF342082 for *XCXCL13L2*, CB199271 for *XCXCLa*, BC130085 for *XCXCLb*, AW644053 for *XCXCLd*, CF548813 for *XCXCLe*, AJ312936 for *XCXCR1/2*, BC157471 for *XCXCR6*. The primers to obtain the full-length cDNA of *XCXCR3* were designed with reference to the

TABLE 2

PRIMER PAIRS USED IN RT-PCR ANALYSIS

Gene	Primer
XCXCL13L1	Forward: 5′-TTGCTGTCCTGCTGTGCTTA-3′ Reverse: 5′-GCTCCAGATCCCTTCCAATT-3′′
XCXCL13L2	Forward: 5′-TGCTGTGCTGTCTGTCATTG-3′ Reverse: 5′-TATTGGTGTACTGTCTGCCG-3′
XCXCLa	Forward: 5′-ACCGTATCAACCGCCTTTCA-3′ Reverse: 5′-GACTGCTGAATGAAAGAGGG-3′
XCXCLb	Forward: 5′-AATCTGGGGAACGTGTGTGT-3′ Reverse: 5′-GAGAATTTGGAAGCATGGCC-3′
XCXCLd	Forward: 5′-GCACGAGGAAATTGAGACCA-3′ Reverse: 5′-TCTCTGTTGCCTGCTTGTAG-3′
XCXCLe	Forward: 5′-GGAAACCAAGAGAAGTGTCC-3′ Reverse: 5′-CAAGCAGGTTCCACTCAATG-3′
XCXCR1/2	Forward: 5′-GACACTTGGTAGGCTTCTTC-3′ Reverse: 5′-AGCATAACGGGCCAGAAAAG-3′
XCXCR3	Forward: 5′-GAAACAGAAAGCCCTACGAG-3′ Reverse: 5′-AGTTGAAGGTTTGGCTGGAG-3′
XCXCR5	Forward: 5′-GGGATTCTGCTATGCTCACA-3′ Reverse: 5′-CCATTTTCAGAGTCAGTGGC-3′
XCXCR6	Forward: 5′-ACTCTCACCGACACCTTCAT-3′ Reverse: 5′-GCAAGTAACAGCGACACCAA-3′
XCXCRa	Forward: 5′-TAGCCTGCATTGGACTGAAC-3′ Reverse: 5′-TTGAGAACGGTAGGAGTGAC-3′
Ornithine decarboxylase (*ODC*)	Forward: 5′-AAAATGGATGACTGCGAGATGGG-3′ Reverse: 5′-AATGAAGATGCTGACTGGCAAAAC-3′

nucleotide sequence of BC073571. There are 3-amino acid mismatches between XCXCR3 and the translated sequence of BC073571. The primers to obtain the full-length cDNA of *XCXCR5* were designed with reference to the nucleotide sequences of BX845251 for 5'-region and BE132148 for 3'-region of *XCXCR5*. The primers to obtain the full-length cDNA of *XCXCRa* were designed with reference to the nucleotide sequences of BX850143 for 5'-region and BI313710 for 3'-region of *XCXCRa*. The cDNA fragments were subcloned into a modified pCS2+ vector as previously reported (Goto *et al*., 2008).

RT-PCR analyses

Total RNA was prepared using TRIzol (Invitrogen). cDNA synthesis was carried out using Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR was performed using rTaq DNA polymerase (TAKARA). Table 2 lists the primer pairs used for RT-PCR analyses of expression patterns of CXC-type chemokine ligands and receptors, which were performed as previously described (Suzawa *et al*., 2007) at least in triplicate. *Xenopus* embryonic *Ornithine decarboxylase (ODC)* was used for normalization of cDNA samples. For RT-PCR analyses of mesodermal marker genes, the sequences of the primer pairs were as follows. *Chordin*: Forward 5'-TTTCCTGTACCAACCCAATCC-3'; Reverse 5'-GGCAGGATTTAGAGTTGCTTC-3'. *Xbra*: Forward 5'-ATAGCAGTGACCGCATACCAG-3'; Reverse 5'-GCTGGCATTTGAAGGGTAGAC-3'. *MyoD*: Forward 5'-CCGGTTCTGGAACATTACAG-3'; Reverse 5'-AGGGGAAGTTCATGGATTGG-3'.

Microinjection of mRNAs

Capped mRNAs were synthesized from linearized vectors using Thermo T7 RNA polymerase (TOYOBO). The mRNA of each *Xenopus* chemokine ligand was microinjected into the marginal zones of two dorsal or ventral blastomeres of 4-cell embryos (500 pg/blastomere).

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