Developmental expression and regulation of the chemokine CXCL14 in Xenopus

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ABSTRACT Chemokines are a family of proteins originally identified for their activity promoting the recruitment of leukocytes to inflammatory sites. Recent evidence indicates that chemokines and their receptors may also regulate key developmental processes. In this paper we report the expression and regulation of the chemokine CXCL14 during Xenopus laevis embryogenesis. CXCL14 is first detected in several ectoderm derivatives, the dorsal aspect of the retina, the cement gland and the hatching gland. Later in development, additional domains of expression include the head mesenchyme and the medial ventral aspect of the otic vesicle. CXCL14 expression in the ectoderm is regulated by both Bmp and canonical Wnt signaling. In the hatching gland CXCL14 is co-expressed with the transcription factor Pax3. Using gain of function and knockdown approaches in whole embryos and animal explants we show that Pax3 is both necessary and sufficient for CXCL14 expression in this domain of the ectoderm.

KEY WORDS: CXCL, hatching gland, cement gland, otic vesicle, retina

Introduction

Chemokines form a family of chemotactic cytokines initially identified by their ability to regulate leukocyte trafficking during inflammatory response. They are classified into four groups, CCL, CXCL, XCL and CX3CL, based on the number and spacing of conserved cysteine (C) residues within their sequence. Chemokines mediate their biological effects through interaction with G protein-coupled receptors. These receptors are referred as CCR, CXCR, XCR and CX3CR based on the chemokine subclass by which they are activated (Zlotnik and Yoshie, 2000).

Chemokines of the CXCL class are best known for promoting directional cellular migration of leukocytes during inflammation (Zlotnik and Yoshie, 2000). They also affect tumor development by influencing angiogenesis, tumor transformation, growth and invasion (Vandercappellen et al., 2008). The up-regulation of several chemokines and their receptors in the adult brain has been implicated in a number of neurodegenerative diseases, such as multiple sclerosis, Alzheimer’s disease and dementia (Cartier et al., 2005). A number of recent reports indicate that chemokines may also regulate directional migration of cells in other contexts besides the inflammatory response, including embryogenesis. For example, in the zebrafish disruption of the chemokine CXCL12 (also known as SDF-1) or its receptor CXCR4a cause the endoderm to separate from the mesoderm and as a result the endoderm migrates more anteriorly than normal, leading to the duplication of endodermal organs (Nair and Schilling, 2008; Mizoguchi et al., 2008). In the mouse embryonic olfactory system, SDF-1/CXCR4 signaling influences sensory axon extension and the migration of gonadotropin releasing hormone-1 neurons (Toba et al., 2008). Signaling through CXCL12/CXCR4b and CXCL12/CXCR7 controls the migration of primordial germ cells in zebrafish and medaka embryos (Boldajpour et al., 2008; Sasado et al., 2008). CXCR7 function has also been implicated in the proper migration of the zebrafish posterior lateral line primordium (Valentin et al., 2007; Dambly-Chaudiere et al., 2007). In Xenopus embryos SDF1/CXCR4 signaling has been proposed to control the directional movement of mesendodermal cells during gastrulation (Fukui et al., 2007). Altogether these studies highlight the importance of this class of molecules during early embryonic development.

Abbreviations used in this paper: SDF-1, stromal cell-derived factor-1; Trp-2, Tryosinase related protein-2.
of this class of molecules, are underlined. Conserved cysteine residues, signature motif using GeneDoc. Conserved amino acids in all five species or in at least two species are highlighted in black and grey, respectively. The conserved cysteine residues, signature motif

Results & Discussion

Sequence and developmental expression of Xenopus CXCL14

An 1160bp PCR product containing the entire ORF of CXCL14 was amplified from stage 30 cDNA. Sequence analysis (Fig 1A) indicates that at the amino acid level, CXCL14 shares 68% identity with human CXCL14, also known as BRAK, (Hromas et al., 1999), 67% identity with mouse CXCL14 (Hromas et al., 1999), 58% identity with chicken CXCL14 (accession # NP990043) and 65% with zebrafish Scyba (Long et al., 2000). Gene synteny analysis (not shown) provides further evidence that the Xenopus gene is the true ortholog of CXCL14.

There is no significant homology between Xenopus CXCL14 and the only other Xenopus CXCL family member isolated to date, SDF-1 (Braun et al., 2002). RT-PCR analysis of the developmental expression of CXCL14 reveals that this chemokine is not maternally expressed in Xenopus. It is first detected at stage 20 and persists throughout development, at least up to stage 40 (Fig 1B). For comparison Xenopus SDF-1 is activated around the same stage (stage 18/20; Braun et al., 2002) while its receptor CXCR4 is maternally expressed and up regulated after the mid-blastula transition (Fukui et al., 2007). CXCL14 is among a small number of chemokines with no known receptor.

Spatial expression of Xenopus CXCL14

To analyze the expression of Xenopus CXCL14, whole-mount in situ hybridization was performed on embryos at different stages. CXCL14 transcripts were first detected at stage 20 in a region anterior to the neural plate corresponding to the prospective cement gland (Fig 2A). By stage 23 CXCL14 is detected in three different domains of the ectoderm the cement gland, the hatching gland and the dorsal anterior part of the optic vesicle (Fig 2B-D). At this stage CXCL14 expression appears to be restricted to the anterior half of the cement gland (Fig 2C). The hatching gland is a specialized group of cells that produces proteolytic enzymes, involved in the digestion of the vitelline envelope and jelly coat (Drysdale and Elison, 1991). In this tissue CXCL14 is co-expressed with Xhe (Xenopus hatching enzyme; Fig 2E) and the transcription factor Pax3 (Fig 2F). At stage 28, CXCL14 persists in all three domains of the ectoderm and its expression is also initiated in the otic vesicle (Fig 2G-I). CXCL14 is expressed in the dorsal portion of the retina, reminiscent to Trp2, a pigment cell-specific enzyme involved in melanin synthesis (Fig 2J; Aoki et al., 2003), suggesting that CXCL14 expression is confined to the pigmented epithelium of the retina. Transverse sections through stage 28 embryos confirm that CXCL14 is indeed confined to this layer of the retina (Fig 2K, L). In the developing inner ear CXCL14 expression is restricted to the ventral medial aspect of the otic vesicle (Fig 2M). Later in development (stage 35) CXCL14 expression persists strongly in the pigmented epithelium of the retina dorsally, and the otic vesicle while its expression in the cement gland and the hatching gland starts to fade (Fig 2N-Q). At this stage CXCL14 is also detected in the head mesenchyme (Fig 2N, O). In the otic vesicle CXCL14 expression is distinct and medial to that of Otx2, a transcription factor that is confined to the ventral aspect of the otocyst (Saint-Germain et al., 2004; Fig 2R, S). Anatomically the otic expression of CXCL14 overlaps with a region of the otocyst from which the vestibulo-accoustic ganglia derives.

The zebrafish CXCL14, also known as Scyba, is primarily expressed in the midbrain region and the otic placode at early stages, and later in a subset of hindbrain neurons, the sensory patches of the otic vesicle and the lateral line (Long et al., 2000). While CXCL14 is expressed in derivatives of the ectoderm in both fish and frog, the only common domain of expression of CXCL14 in both species is the otic vesicle. The divergence in the expression of CXCL14 suggests that the expression pattern and presumably the function of this chemokine may have been altered during evolution. So far the embryonic expression of CXCL14 has not been described in amniotes.

Bmp and canonical Wnt signaling regulate CXCL14 expression in the ectoderm

Bmp activity alone or in combination with canonical Wnt signaling has been implicated in
Fig. 3. CXCL14 expression in the ectoderm is regulated by both Bmp and canonical Wnt signaling. Embryos at the 2-cell stage were injected in the animal pole with two distinct doses of Noggin (N) mRNA (0.4 ng and 1.0 ng) alone or in combination with Wnt1 (+W) mRNA (0.1 ng). Animal explants were dissected at the blastula stage, cultured for 10 hours and analyzed by Real-Time RT-PCR for the activation genes expressed in various domains of the ectoderm. Un, uninjected animal explant. Each value has been normalized to the level of EF-1α expression.
the generation of multiple cell lineages in the ectoderm, including the neural crest, the hatching gland, pre-placodal ectoderm, neural plate, cement gland and epidermis (reviewed in Knecht and Bronner-Fraser, 2002; Huang and Saint-Jeannet, 2004). Each one of these cell populations appears to form in response to specific thresholds of Bmp activity (Hong and Saint-Jeannet, 2007). To analyze the regulation of CXCL14 expression in the ectoderm, embryos were injected with two doses of Pax3 overexpression (0.5 ng) in the whole embryo results in a dramatic expansion of CXCL14 and Xhe expression domains at stage 23 (arrows), while Snail2 expression is reduced at stage 15 (arrow). For the embryos hybridized with CXCL14 or Xhe, anterior view is shown, dorsal to top. For Snail2 the embryos are viewed from the dorsal side anterior to top. In animal explants CXCL14 and Xhe are activated by higher doses of Pax3GR mRNA as compared to Snail2. The concentrations of injected Pax3GR mRNA are 0.05 ng, 0.1 ng, 0.25 ng, 0.5 ng and 1 ng. Each Real-Time RT-PCR value has been normalized to the level of EF-1α expression.

**Fig. 4. Pax3 is necessary and sufficient for CXCL14 expression in the hatching gland.** (A) Embryos injected with Pax3 morpholino (Pax3MO; 40 ng) exhibit a strong reduction of CXCL14 expression in the hatching gland (arrow). In these Pax3-depleted embryos Snail2 and Xhe expression is also reduced at the neurula and tailbud stages, respectively (arrows). (B) Pax3 overexpression (0.5 ng) in the whole embryo results in a dramatic expansion of CXCL14 and Xhe expression domains at stage 23 (arrows), while Snail2 expression is reduced at stage 15 (arrow). For the embryos hybridized with CXCL14 or Xhe, anterior view is shown, dorsal to top. For Snail2 the embryos are viewed from the dorsal side anterior to top. (C) In animal explants CXCL14 and Xhe are activated by higher doses of Pax3GR mRNA as compared to Snail2. The concentrations of injected Pax3GR mRNA are 0.05 ng, 0.1 ng, 0.25 ng, 0.5 ng and 1 ng. Each Real-Time RT-PCR value has been normalized to the level of EF-1α expression.

and hatching gland (Xhe) fate required Bmp attenuation as well as active Wnt signaling (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; McGrew et al., 1999). While activation of neural crest- and hatching gland-specific genes occurs in response to the same combination of signals, hatching gland fate requires a higher level of Bmp inhibition (Fig 3). In this assay CXCL14 follows a pattern of activation similar to that of Xhe and Pax3 (Fig 3; Hong and Saint-Jeannet, 2007), consistent with CXCL14 expression in the developing hatching gland (Fig 2). As expected in these explants the neural plate-specific gene (Sox2) was up regulated by single injection of Noggin mRNA, and associated with strong repression of epidermal fate (Keratin). These results indicate that CXCL14 expression in the ectoderm is largely dependent on the combined activity of Bmp and canonical Wnt signaling.

**Pax3 is necessary and sufficient for CXCL14 expression**

Pax3 has been shown to be required for hatching gland formation in Xenopus (Hong and Saint-Jeannet, 2007). To determine whether Pax3 was also involved in controlling CXCL14 expression is this region of the ectoderm, we used a Pax3-specific morpholino oligonucleotide (Pax3MO), which blocks Pax3 translation (Monsoro-Burq et al., 2005). Unilateral injection of Pax3MO at the 2-cell stage inhibited Xhe (96%; n=54) and Snail2 (100%; n=22) expression (Fig 4A) as previously reported (Monsoro-Burq et al., 2005; Sato et al., 2005; Hong and Saint-Jeannet, 2007). Pax3 knockdown also prevented CXCL14 expression in the hatching gland (98%; n=46) of tailbud stage embryos (Fig 4A).

**CXCL14 expression in the cement gland and the dorsal aspect of the retina was also affected in some of these embryos, an effect that is likely to be secondary to the craniofacial defects associated with the loss of neural crest tissue in Pax3 morphants (Monsoro-Burq et al., 2005).** These results demonstrate that Pax3 is required upstream of CXCL14 in the developing hatching gland.

We have previously shown that Snail2 induction in the embryo depends on a specific level of Pax3 activity, and that excess Pax3 activity in the ectoderm is incompatible with neural crest development, but rather promotes hatching gland fate (Hong and Saint-Jeannet, 2007). We therefore decided to test whether CXCL14 was also dependent on the level of Pax3 activity in the ectoderm. We found that the dose of Pax3GR mRNA (0.5 ng) that induces ectopic Xhe expression (100% of the embryos; n=53) and represses Snail2 expression at the neural plate border (97% of the embryos; n=30), also promoted a dramatic expansion of
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CXCL14 expression domain (Fig 4B) in 100% of injected embryos (n=58). To confirm these observations we monitored by Real-Time RT-PCR the induction of CXCL14 in animal explants derived from embryos injected with increasing doses of Pax3GR mRNA (0.05 ng - 1 ng) and cultured in the presence of dexamethasone for 10 hours. As previously described, we found that neural crest (Snail2) and hatching gland (Xhe) fates were specified by different levels of Pax3 activity (Fig 4C; Hong and Saint-Jeannet, 2007). While Snail2 expression is mildly activated by intermediate doses of Pax3GR mRNA (0.25 ng), Xhe is induced by a broader range of Pax3GR mRNA, including the higher dose (1 ng). In these explants the CXCL14 induction profile is more similar to that of Xhe, with maximum level of induction observed for 0.5 ng of Pax3GR mRNA (Fig 4C).

Altogether these findings indicate that Pax3 is not only required but is also sufficient for CXCL14 expression in the ectoderm and suggests that its expression in the developing hatching gland depends on specific levels of Pax3 activity.

Materials and Methods

Isolation of Xenopus CXCL14

Xenopus CXCL14 was amplified by PCR from stage 30 cDNA using specific primers (F: CGTGGAGCTTGCTCTCTTT and R: GTGCGCAAGAATGGGTTAAA) based on the sequence available in GenBank (accession # BC074316). The PCR condition were as follow: denaturation at 94 °C (30 sec.), annealing at 55 °C (30 sec.), and extension at 72 °C (90 sec.) for 35 cycles. The resulting 1160 bp PCR product was purified, subcloned into pGEMTeasy (Promega) and sequenced. This construct is referred as sec.), annealing at 55 °C (3 sec.), and extension at 72 °C (12 sec.). By optimizing primers and reaction conditions, a single specific product was amplified as confirmed by melting curve analysis. Each reaction included a control without template and a standard curve of serial dilutions (in 10-fold increments) of test RNAs. In each case, elongation factor 1α (EF1α) was used as an internal reference (data not shown). Each bar on the histograms has been normalized to the level of EF1α.

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