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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 knows 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

Abbreviations used in this paper: SDF-1, stromal cell-derived factor-1; Trp-2, Tryosinase related protein-2.

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development and organogenesis.

We have initiated the characterization of a number of chemokines in the frog *Xenopus laevis* to analyze the function of this class of molecules during embryogenesis. Here we report the expression of *CXCL14* in the embryonic ectoderm and its derivatives and analyze the regulatory inputs that control aspects of *CXCL14* expression in this germ layer.

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 Elinson, 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.

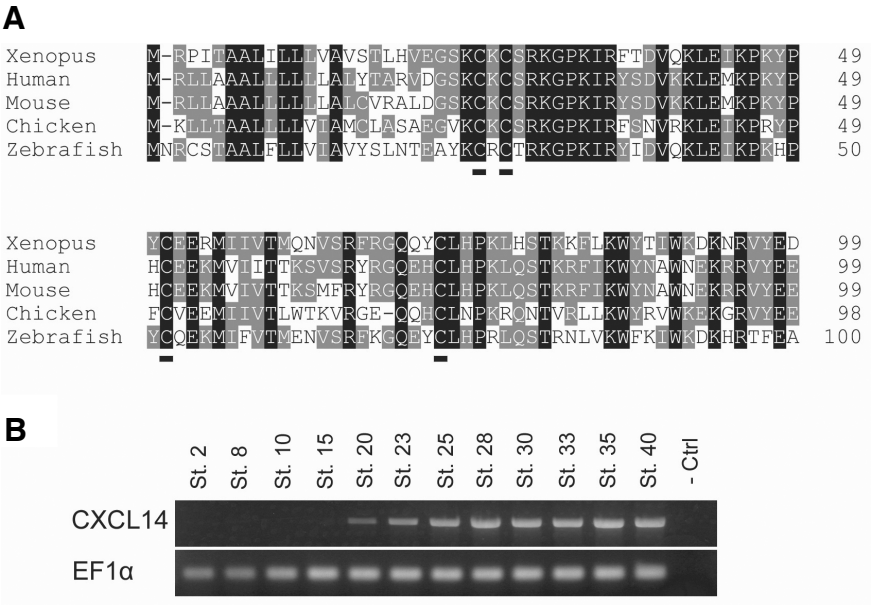

Fig. 1. Sequence and developmental expression of *Xenopus* CXCL14. (A) *Deduced amino acid sequences from human, mouse, chicken and zebrafish CXCL14 were aligned using GeneDoc. Conserved amino acids in all five species or in at least two species are highlighted in black and grey, respectively. The conserved cysteine residues, signature motif of this class of molecules, are underlined.* **(B)** *RT-PCR analysis of the developmental expression of* CXCL14. *Stages are according to Nieuwkoop and Faber (1967). EF1α is shown as a loading control.*

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

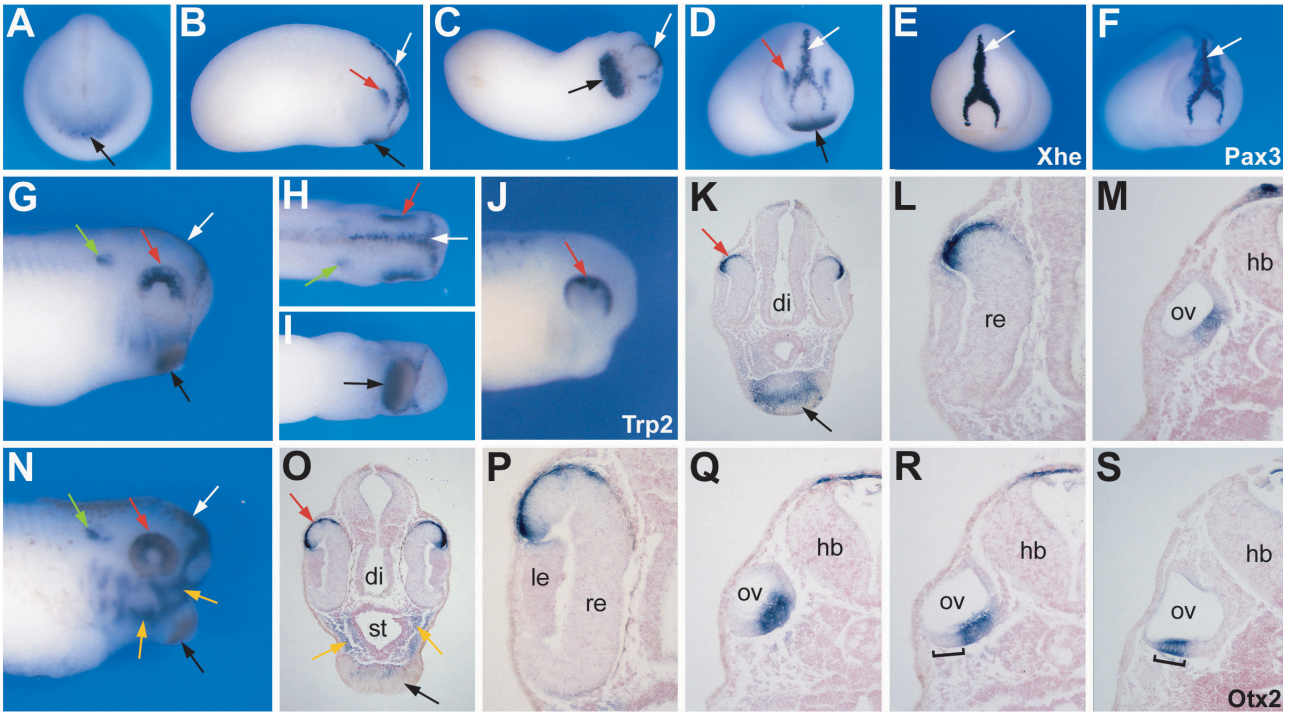

Fig. 2. Expression of *CXCL14* by whole-mount *in situ* hybridization. (A) *Onset of CXCL14 expression at stage 20 in the prospective cement gland anterior to the neural plate (black arrow). Frontal view, dorsal to top.* **(B-D)** *At stage 23* CXCL14 *is detected in part of the cement gland (black arrows), the dorsal anterior portion of the optic vesicle (red arrows) and the hatching gland (white arrows).* **(B)** *Lateral view, dorsal to top, anterior to right.* **(C)** *Ventral view, anterior to right.* **(D)** *Frontal view, dorsal to top. The expression of* Xhe **(E)** *and* Pax3 **(F)** *in the hatching gland (white arrows) at this stage are shown for comparison. (E-F) Frontal view, dorsal to top.* **(G-I)** *CXCL14 expression at stage 28. In addition to the cement gland (black arrows), optic vesicle (red arrows) and hatching gland (white arrows),* CXCL14 *is also detected in the otic vesicle (green arrows).* **(G)** *Lateral view, dorsal to top, anterior to right.* **(H)** *Dorsal view, anterior to right.* **(I)** *Ventral view, anterior to right.***(J)** *The expression pattern of* Trp2 *in the pigmented epithelium of the retina is similar to* CXCL14 *expression in the developing eye (red arrow). Lateral view, dorsal to top, anterior to right.* **(K-L)** *Transverse section highlights the expression of* CXCL14 *expression in the pigmented epithelium of the retina (red arrow). Panel (L) is a higher magnification view of panel* **(K)**. *CXCL14 expression is restricted to the ventral medial aspect of the otic vesicle* **(M)**. **(N)** *At stage 35, CXCL14 expression persists in the same domains and is also detected in the head mesenchyme (yellow arrows). Lateral view, dorsal to top, anterior to right.* **(O-R)** *Transverse sections showing the expression of* CXCL14 *in the head mesenchyme and the cement gland* **(O)**, *pigmented epithelium of the retina* **(O-P)** *and otic vesicle* **(Q-R)**. *Panel (Q) corresponds to a section posterior to section shown in panel (R). For comparison at this stage* Otx2 *(bracket) is expressed in the ventral aspect of the otic vesicle* **(S)** *a domain that does not overlap with* CXCL14 *(R). di, diencephalon; hb, hindbrain; le, lens; ov, otic vesicle; re, retina; st, stomodeum.*

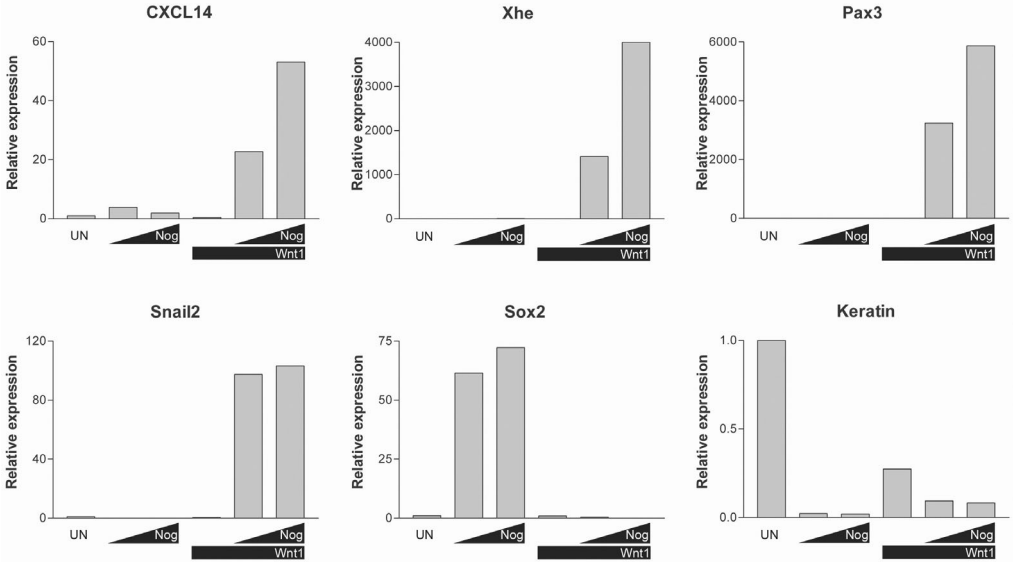

Fig. 3. *CXCL14* expression in the ectoderm is regulated by both Bmp and canonical Wnt signaling. *Embryos at the 2-cell stage were injected in the animal pole with two distinct doses of* Noggin *(N) mRNA (0.4 ng and 1.0 ng) alone or in combination with* Wnt1 *(+W) mRNA (0.1 ng). Animal explants were 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.*

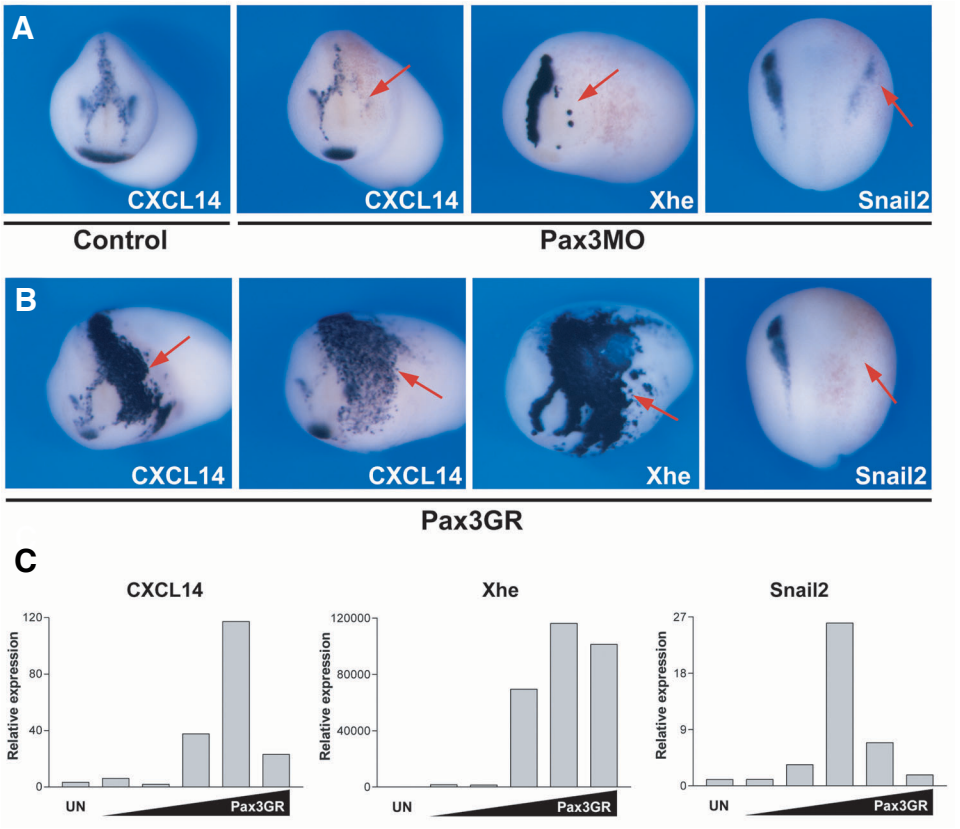

Fig. 4. Pax3 is necessary and sufficient for *CXCL14* expression in the hatching gland. (A) *Embryos injected with Pax3 morpholino (Pax3MO; 40ng) exhibit a strong reduction of* CXCL14 *expression in the hatching gland (arrow). In these Pax3-depleted embryos* Snail2 *and* Xhe *expression is also reduced 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 1ng. Each Real-Time RT-PCR 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 *Noggin* mRNA alone (to modulate the level of Bmp activity), or in combination with *Wnt1* mRNA. The corresponding animal explants were isolated at the blastula stage, cultured for 10 hours (equivalent stage 15) and analyzed by Real-Time RT-PCR. In these explants the expression of *CXCL14* was compared to the expression of genes expressed in various domains of the ectoderm, including *Pax3* (neural plate border and hatching gland; Bang *et al.*, 1997), *Snail2* (neural crest; Mayor *et al.*, 1995), *Xhe* (hatching gland; Katagiri *et al.*, 1997), *Sox2* (neural plate; Mizuseki *et al.*, 1998), and *Keratin* (epidermis; Jonas *et al.*, 1989).

As previously reported induction of both, neural crest (*Snail2*) 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

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: CGTGGAGCTTGCTCTCTCTT and R: GTGGGCAAGAATGGGTTAAA) based on the sequence available in GenBank (accession # BC074316). The PCR condition were 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 pGEMT-CXCL14. The 1160bp PCR product contains the entire ORF of *CXCL14* (300 bp), 112bp of 5'UTR and 748 bp of 3'UTR. Gene synteny analysis of human, mouse, chicken, zebrafish and *Xenopus CXCL14* was performed using Metazome (http://www.metazome.net).

Xenopus *embryo manipulation and injections*

Embryos were staged according to Nieuwkoop and Faber (1967). *Wnt1* (0.1 ng; Wolda *et al*., 1993), *noggin* (0.4 ng and 1 ng; Smith and Harland, 1992) and *Pax3GR* (0.1 ng - 1 ng; Hong and Saint-Jeannet, 2007) mRNAs were synthesized *in vitro* using the Message Machine kit (Ambion, Austin, TX). Pax3 morpholino antisense oligonucleotide (Pax3MO; 40ng; Monsoro-Burq *et al*., 2005) was purchased from Gene-Tools (Philomath, OR). In whole embryo experiments, Pax3GR mRNA and Pax3MO were injected in the animal pole of one blastomere at the two-cell stage and analyzed by *in situ* hybridization at stage 15 *(Snail2)* and stage 23 (*CXCL14* and *Xhe*). Embryos injected with *Pax3GR* were treated with 10 μM dexamethasone (Sigma) at stage 11. Sibling injected embryos cultured in the absence of dexamethasone were used as control. For animal explants, both blastomeres of two-cell stage embryos were injected with mRNAs in the animal pole region and animal caps were dissected at the late blastula stage and immediately cultured *in vitro* in 0.5X NAM (Normal Amphibian Medium; Slack and Forman, 1980). Animal caps from *Pax3GR*-injected embryos were cultured in 0.5X NAM supplemented with 10 μM of dexamethasone. After 10 hours in culture, animal explants were analyzed by Real-Time RT-PCR (Roche Diagnostics) for the expression of various marker genes (Hong and Saint-Jeannet, 2007; Park and Saint-Jeannet, 2008).

Whole-mount in situ *hybridization*

In all experiments, blastomeres were co-injected with 1 ng of β-*galactosidase* mRNA (β-gal, 1 ng) to identify the injected side. At stage 15 or 23 embryos were fixed in MEMFA (Harland, 1991) and successively processed for Red-Gal (Research Organics) staining and *in situ* hybridization. Antisense DIG-labeled probes (Genius kit, Roche) were synthesized using template cDNA encoding *Xhe* (Katagiri *et al*., 1997), *Snail2* (Mayor *et al*., 1995), *Trp2* (Aoki *et al*., 2003), *Pax3* (Bang *et al*., 1997), *Otx2* (Pannese *et al*., 1995) and *CXCL14* (pGEMT-CXCL14). Whole-mount *in situ* hybridization was performed as described (Harland, 1991). For histology, stained embryos were embedded into Paraplast+, 12 μm sections cut on a rotary microtome and counterstained with Eosin.

Real-time RT-PCR

For each sample, total RNAs were extracted from 10 animal caps using an RNeasy microRNA isolation kit (QIAGEN, Valencia, CA) according to the manufacturer's direction. During the extraction procedure the samples were treated with DNase I, to eliminate possible contamination by genomic DNA. The amount of RNA isolated from tissues was quantified by measuring the optical density using a spectrophotometer (Beckman Coulter, Fullerton, CA). Real-time RT-PCR was performed using specific primers (Hong and Saint-Jeannet, 2007; Park and Saint-Jeannet, 2008) and the QuantiTect SYBR Green RT-PCR kit (QIAGEN) on a LightCycler (Roche Diagnostics). The primers for *CXCL14* were as follow, F: ATGGAAGATGCCAGACAAGG and R: TTCAATGCCAGAATGTTGA. The reaction mixture consisted of 10 μl of QuantiTect SYBR Green RT-PCR Master Mix, 500 nM forward and reverse primers, 0.2 μl of RT, and 60 ng of template RNA in a total volume of 20 μl. The cycling conditions were as follow: denaturation at 95°C (3 sec.), annealing at 55°C (4 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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