An essential role of *Xenopus Foxi1a* for ventral specification of the cephalic ectoderm during gastrulation

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Summary

During gastrulation in *Xenopus*, the head ectoderm is subdivided into the central nervous system (CNS) anlage (neural plate) and the non-CNS ectoderm (i.e. epidermis, placodes and neural crest). The winged-helix transcription factor Xfoxi1a is one of the earliest markers for the preplacodal region at the mid-neurula stage. Interestingly, before the establishment of the preplacodal region, *Xfoxi1a* expression is detected in the entire cephalic non-neural ectoderm at the mid- and late gastrula stages. The present study focuses on the role of Xfoxi1a particularly at the gastrula stages. The early *Xfoxi1a* expression in the anteroventral ectoderm is dependent on Bmp signals and suppressed by Wnt signals. Inhibition of *Xfoxi1a* activities by injection of antisense oligonucleotides leads to suppression of non-CNS ectodermal markers (e.g. *keratin*) and expansion of the anterior expression domain of the CNS marker *Sox2*. Conversely, misexpression of *Xfoxi1a* suppresses *Sox2* and induces *keratin* in the anterior neural plate. In the animal cap, *Xfoxi1a* overexpression antagonizes the neuralizing activity of Chordin (Chd). Studies using an inducible *Xfoxi1a* construct (*GR-Xfoxi1a*) show that the ventralizing function of *Xfoxi1a* is confined to the gastrula stage. Thus, *Xfoxi1a* is an essential regulator of ventral specification of the early head ectoderm during gastrulation.

Key words: *Xenopus*, Foxi1a, Ectoderm, CNS

Introduction

In vertebrate gastrula embryos, the ectoderm is subdivided into various regional tissues by complex inductive processes. Along the dorsoventral (DV) axis, the ectoderm becomes subdivided into the dorsal (CNS or neural plate), intermediate (e.g. presumptive neural crest, placodes and cement gland) and ventral (epidermal) ectoderm. Although it is generally believed that the early DV specification is controlled by a Bmp activity gradient in *Xenopus* (Wilson et al., 1997; LaBonne and Broner-Fraser, 1998; Marchant et al., 1998; Tríbulo et al., 2003), how the exact subdivision boundaries are determined remains largely elusive.

Along the anteroposterior (AP) axis, the neural plate is finely regionalized into the forebrain, midbrain, hindbrain and spinal cord. Recent molecular studies implicated Wnts, Nodal, Fgfs and RA in the AP regionalization of the CNS (McGrew et al., 1995; Kengaku and Okamoto, 1995; Piccolo et al., 1999; Gavalas and Krumlauf, 2000; Kiecker and Niehrs, 2001; Kudoh et al., 2002; Onai et al., 2004).

In contrast to the CNS, relatively little is known about the AP specification of the non-CNS (intermediate and epidermal) ectoderm. In *Xenopus*, the non-CNS ectoderm of the head region (referred to as 'cephalic non-neural ectoderm' hereafter) has several characteristic features. For example, unlike that of the trunk, the non-neural ectoderm of the cephalic region differentiates into the cranial placodes and special exocrine glands such as the cement gland and hatching gland, in addition to the epidermis and neural crest. The cranial placodes, which develop within the preplacodal field of the head intermediate ectoderm, give rise to a number of sensory tissues (reviewed by Baker and Bronner-Fraser, 2001). To date, the molecular mechanism underlying the determination of the cephalic non-neural ectoderm (versus the CNS and the trunk ectoderm) remains largely to be elucidated.

In this study, we have investigated the molecular control of the initial specification of the cephalic non-neural ectoderm by focusing on the roles of a Foxi1 family gene in *Xenopus*. The winged-helix transcription factor Foxi1 plays an essential role for the formation of placode-derived ectodermal tissues such as the otic vesicle (Hulander et al., 1998; Nissen et al., 2003; Solomon et al., 2003a) in mice and zebrafish. In *Xenopus*, three Foxi1-related genes have been reported: *Xfoxi1a*, *Xfoxi1b* (pseudoalleles generated by the pseudotetraploidy of *Xenopus laevis*, see alignment of *Xfoxi1a* and *Xfoxi1b* in Fig. 1M) and *Xfoxi1c* (which is not an *Xfoxi1a* pseudoallele) are expressed in the preplacodal area at the neurula stage (Lef et al., 1994; Pohl et al., 2002).

Interestingly, *Xfoxi1a* and *Xfoxi1b* are also expressed even earlier than the establishment of the preplacodal expression at the neurula stage; they are expressed widely in the animal side of the embryo at the late blastula stage and in the anteroventral ectoderm at the late gastrula stage. By contrast, *Xfoxi1c* is expressed only after the gastrula stage and not during the blastula and gastrula stages (Pohl et al., 2002). The expression of a *foxi1* gene in a broad domain of the gastrula ectoderm has been reported also in zebrafish (Nissen et al., 2003; Riley and

Phillips, 2003; Solomon et al., 2003a). However, the role of the Foxi1 family genes during the gastrula stage has not yet been elucidated. In addition, although several transcription factors have been implicated in the development of the non-neural ectoderm in *Xenopus* (e.g. Dlx3, Msx1, Gata1 and Xvent1/2) (Onichtchouk et al., 1996; Suzuki et al., 1997; Ault et al., 1997; Onichtchouk et al., 1998; Feledy et al., 1999; Beanan and Sargent, 2000; Woda et al., 2003), none of them are expressed in a pattern limited to the cephalic non-neural ectoderm during gastrulation. These facts led us to investigate the role of *Xfoxi1a* (including that of the *Xfoxi1b*; the term *Xfoxi1a/b* is used hereafter when the combined functions are considered) in the head ectoderm of the *Xenopus* gastrula. By focusing on the role at the early stage, we demonstrate that *Xfoxi1a/b* is essential for the specification of the non-neural ectoderm in the head. We also show that *Xfoxi1a/b* misexpression promotes epidermal differentiation at the cost of neural tissues. We discuss a possible mode of the *Xfoxi1a/b* action, focusing on the critical period of *Xfoxi1a/b*-mediated ectodermal patterning.

Materials and methods

Plasmid construction

The coding sequence of *Xfoxi1a* (GenBank Accession Number X74315) was amplified from *Xenopus* stage 12.5 cDNA by PCR using KOD Plus DNA polymerase (Toyobo, Osaka, Japan). The resulting cDNA was subcloned into the *Eco*RI-*Xho*I site of the *pCS2* vector (Turner and Weintraub, 1994). The ligand-binding domain of the glucocorticoid receptor (Hollenberg et al., 1985; Hollenberg et al., 1993; Kolm and Sive, 1995) was amplified by PCR with these primers: forward 5′-GCCGGATCCACCATGACCTCTGAAAATCC-3′ and reverse 5′-GCCATCGATCCTTTTGATGAAACAGAAG-3′. The resulting products were fused in frame at the *Bam*HI-*Cla*I site in the above plasmid (*Xfoxi1a/pCS2*). The *Xfoxi1a* and *Xfoxi1b* (GenBank Accession Number X74316) with the 5′-UTR sequence and an additional C-terminal flag-tag sequence were constructed by using the following primers: forward 5′-GCCATCGATT-CAGTTGGGAAAGAGCAGAAGCCGCTG-3′ and reverse 5′-GCCCTCGAGTTACTTATCGTCGTCATCCTTGTAATCGTACCT-TCCCTGGTACAGAGGAGACCTGC-3′; forward 5′-GCCATCGA-TTCTGCATCAGTTAGAAAAGAGCGATT-3′ and reverse 5′-GCCCTCGAGTTACTTATCGTCGTCATCCTTGTAATCATACCTT-CCCTGGTACAAAGGGGG-3′, respectively. The products were inserted in to the *Cla*I-*Xho*I site of *pCS2*.

Embryonic manipulations

Eggs were collected from adult *Xenopus laevis* and fertilized in vitro as described previously (Sasai et al., 2001). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). After dejellying the embryo by treatment with 2% cysteine (pH 7.8), microinjection was carried out in 1× Barth's solution. Embryos were grown in 0.1× Barth's solution until sibling embryos reached the desired stage. For animal cap assays, ectodermal explants were excised at stage 9 and then cultured in 1× LCMR supplemented with 0.2% BSA until the stages mentioned. For the treatment of the embryo with dexamethasone (Dex), Dex was added to the 0.1× Barth's solution to a 10 μM final concentration at stage 11 or 13, as described by Gammill and Sive (Gammill and Sive, 1997). The embryos were harvested at the neurula stage.

Microinjection and whole-mount in situ hybridization

Capped mRNAs for the microinjection were synthesized by using an SP6 *mMassage* Machine kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA was injected into all animal blastomeres or into the unilateral blasomeres of eight-cell embryos. Morpholino antisense oligonucleotides (Gene Tools, Philomath, OR) were designed against the 5′ regions (see Fig. 3A) of *Xfoxi1a* (*Xfoxi1a-MO*, 5′-GATCAGCGGCTTCTGCTCTTTCCCA-3′) and *Xfoxi1b* (*Xfoxi1b-MO*, 5′-GGTTCATCTCGCTCACTGGCTAATC-3′). Oligonucleotides with five mismatches (*5-mis-Xfoxi1a*, 5′-GAT-CAcCGGgTTCTcCTgTTTCgCA-3′; *5-mis-Xfoxi1b*, 5′-GGTTgATg-TCGCTgACTcGCTAtTC-3′) were used as negative controls. For the rescue experiment, wild-type *Xfoxi1a* mRNA lacking the 5′-UTR sequence was co-injected with *Xfoxi1a-MO* (containing no complimentary sequence). After fixing the embryo with MEMFA at the appropriate stage, whole-mount in situ hybridization was performed as described previously (Sasai et al., 2001). For double in situ hybridization, fluorescein-labeled probe was stained with BCIP (Roche, Mannheim, Germany) and digoxigenin-labeled probe was stained with BM-purple (Roche, Germany) or Magenta-Phos (Biosynth, Switzerland). All of the injection experiments were carried out at least twice and gave reproducible results.

RT-PCR analysis

RT-PCR was performed as described previously (Mizuseki et al., 1998; Kuo et al., 1998; Tsuda et al., 2002). The other primers used first in this study were as follows: *Dlx3* (Papalopulu and Kintner, 1993; Dirksen et al., 1994) (forward primer, ATGAGTGGCCCCTAT-GAGAAGAAG; reverse primer, GGTTCTCTGTAATGGACAAA-CGG); *Sox2* (Mizuseki et al., 1998) (forward primer, GAGGA-TGGACACTTATGCCCAC; reverse primer, GGACATGCTGTA-GGTAGGCGA), *Bmp4* (Dale et al., 1992) (forward primer, GCATGTACGGATAAGTCGATC; reverse primer, GATCTCAGAC-TCAACGGCAC), *Xfoxi1a* (Lef et al., 1994) (forward primer, CCAGAACTGAAATCTTAGCAA; reverse primer, TAACAAAGA-TAAAGCCAGAGGT), *MyoD* (Hopwood et al., 1989) (forward primer, AGGTCCAACTGCTCCGACGGCATGAA; reverse primer, AGGAGAGAATCCAGTTGATGGAAACA), *H4* (Perry et al., 1985) (forward primer, CGGGATAACATTCAGGGTATCACT; reverse primer, ATCCATGGCGGTAACTGTCTTCCT).

Western blot

Animal caps were lysed in the extraction buffer [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% NP-40, 1:100 dilution of protease inhibitor cocktail; Cytoskeleton, Denver, CO] and cleared by micro-centrifugation at 20,000 ***g*** for 10 minutes. Aliquots of 10-30 μg proteins were resolved by 10% SDS-PAGE and then blotted on to a PVDF membrane filter (Millipore, MA). For the primary antibody, anti-FLAG M2 mouse monoclonal antibody (1:1000, Sigma) was used. For the secondary antibody, an anti-mouse IgG horseradish peroxidase linked F(ab′)2 fragment (1:5000, Amersham) was used. Signals were detected with ECL reagents (Amersham).

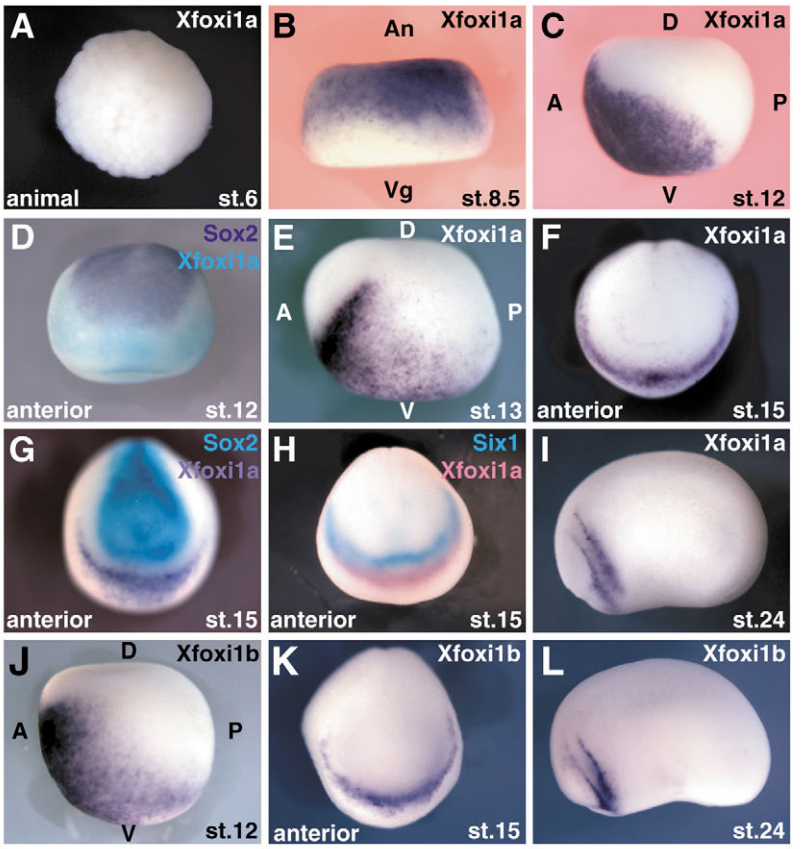
Results

Xenopus Foxi1a is expressed in the anterior-ventral non-neural ectoderm during gastrulation

To understand the role of *Xfoxi1a* during the early steps of embryogenesis (blastula to early neurula), we first performed whole-mount in situ hybridization experiments to analyze the precise pattern of *Xfoxi1a* expression (Fig. 1). Consistent with previous reports (Lef et al., 1994; Pohl et al., 2002), no maternal expression of *Xfoxi1a* was observed (Fig. 1A). *Xfoxi1a* was widely expressed in the animal cap region at the blastula stage (Fig. 1B). Interestingly, *Xfoxi1a* expression was gradually shut off in the dorsal and posterior ectoderm during early gastrulation, and by the mid-gastrula stage it was localized to the anteroventral ectoderm (Fig. 1C). This

expression pattern is consistent with a zebrafish study reporting that a *foxi1* gene is expressed in the anteroventral quadrant of the early gastrula (Nissen et al., 2003; Riley and Phillips, 2003; Solomon et al., 2003a). Double in situ hybridization showed that the expression domains of *Xfoxi1a* and that of *Sox2* (neural plate) (Mizuseki et al., 1998) were complementary to each other and did not overlap (Fig. 1D), indicating that *Xfoxi1a* expression is confined to the cephalic non-neural ectoderm. At the early neurula stage, *Xfoxi1a* expression gradually became limited to the most anterior part of the non-neural ectoderm (Fig. 1E). By the mid-neurula stage, *Xfoxi1a* expression was found only in a horseshoe-shaped domain within the anterior intermediate ectodermal (or preplacodal) region (Fig. 1F). At this stage, an obvious gap was seen between the anterior neural plate and the *Xfoxi1a*$^{+}$ domain (Fig. 1G). This gap area expressed another early preplacodal marker, *Six1* (Pandur and Moody, 2000; Ghanbari et al., 2001) (Fig. 1H), suggesting that the preplacodal region is already divided at the marker level into different DV subdomains by mid-neurulation (*Six1* and *Xfoxi1a* expressions partially overlap in the lateral region but do not coincide in the medial region). Consistent with this idea, double in situ hybridization with a probe for *Xag1* (Sive et al., 1989) indicated that the *Xfoxi1a*$^{+}$ domain (but not the *Six1*$^{+}$ domain) partly overlap with the cement gland anlage (data not shown). At the tailbud stage, *Xfoxi1a* was expressed in restricted branchial arch regions of the head ectoderm (the profundal placodes and the head lateral line system) (Schlosser and Northcutt, 2000) (Fig. 1I) but not in the otic placodes, consistent with a previous study (Pohl et al., 2002).

The expression pattern of the pseudoallele *Xfoxi1b* showed an expression pattern indistinguishable from that of *Xfoxi1a* (tissue distribution at representative stages shown in Fig. 1J-L).



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          **********: *** *: ********************: *:*****:** ***** :**  *******
Xfoxi1a MNPVQQPAQHKCPASSLNPPHPKRAQEAPDMGLYCDNFMYQQHNLHPSHRATNFSIG-DFTHQANPYLWL    69
Xfoxi1b MNPVQQPAQHRSPASLLHLPHPKRAQEAPDMGLYCDNFMFSQQNLHPSQRAPNFSIGGEFTPPANPYLWL    70

          ****:**:* ***:****** *******:*:************************ *************:
Xfoxi1a GGPGVNNSPSYSPTPAPYIPPAFSAPQRQFLANSAAFGGADLGWMSAASQEELLKRVRPPYSYSALIAMS   139
Xfoxi1b GGPGMNNAPNYSPAPAPYIPSAFSAPQRHFMANSAAFGGADLGWMSAASQEELLKMVRPPYSYSALIAMA   140

          ****:************************************************************
Xfoxi1a IQNATDKRLTLSQIYQYVAENFPFYKKSKAGWQNSIRHNLSLNDCFKKMPRDENDPGKGNYWTLDSNCEK   209
Xfoxi1b IQNASDKRLTLSQIYQYVAENFPFYKKSKAGWQNSIRHNLSLNDCFKKMPRDENDPGKGNYWTLDSNCEK   210

          ****************:** *****:***: ************** *: ******** ************
Xfoxi1a MFDNGNFRRKRKPKSETNNIKIAKREEDHVSPKGKESPPMITPSS-PKELSPTGHSKCPSPPTVTYTPCL   278
Xfoxi1b MFDNGNFRRKRKPKSESNNAKIAKRDEDHLNPKGKESPPMITPSSSPEVLSPTGHSKSPSPPTVTYTPCL   280

          ****************** ************: ************** **** **********:     *
Xfoxi1a TNFIGSMTAVDSATMNRQGPLGLLNELSQRNLNGLSSFISGSAVDQSPEHQDSSLFYNRSPYYSSLPTSN   348
Xfoxi1b TNFIGSMTAVDSATMNRQSPLGLLNELSQRNITGLSSFISGSAVDQSSEHQDNSLFYNRSPYYT-----N   345

          **** :******** *******
Xfoxi1a QKQPPYLQQLHPQQSPLYQGRY   370
Xfoxi1b QKQPHFLQQLHPQQPPLYQGRY   367
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Fig. 1. Temporal and spatial expression of *Xfoxi1a/b*. Whole-mount in situ hybridization using albino embryos was performed with *Xfoxi1a* (A-C,E,F,I) or *Xfoxi1b* (J-L) probes. Double in situ hybridization was performed with (D) *Xfoxi1a* (turquoise; BCIP) and *Sox2* (indigo; BM purple) probes, (G) *Xfoxi1a* (indigo; BM purple) and *Sox2* (turquoise; BCIP) probes, and (H) *Xfoxi1a* (purple; magenta-phosphate) and *Xsix1* (turquoise; BCIP) probes. (A) Animal view; (B) lateral view; (C,E,I,J,L) lateral view (anterior towards the left); (D,F,G,H,K) anterior view (dorsal towards the top). The embryo stage is shown in each panel. A, anterior; An, animal; D, dorsal; P, posterior; V, ventral; Vg, vegetal. (M) Amino acid sequence alignment of Xfoxi1a and Xfoxi1b. Identical and similar amino acid residues are marked with asterisks and double dots, respectively. Gaps are indicated by dashes.

Bmp and anti-Wnt signals induce *Xfoxi1a* expression

The in situ hybridization analysis above shows that *Xfoxi1a* is expressed specifically in the anteroventral (or cephalic non-neural) ectoderm during the mid-gastrula and early neurula stages. We next investigated patterning signals that controlled the spatial expression of *Xfoxi1a* during these stages, by focusing on the roles of Bmp and Wnt signals. When *Bmp4* (2.5 pg of the expression plasmid DNA per cell) (Dale et al., 1992) was injected into all animal blastomeres at the eight-cell stage, *Xfoxi1a* expression significantly expanded into the dorsal ectoderm at stage 12 (77%, *n*=13; Fig. 2B), whereas overexpression of the Bmp antagonist *Chd* (50 pg RNA/cell) (Sasai et al., 1995) suppressed *Xfoxi1a* expression (83%, *n*=12; Fig. 2C). We then performed experiments using the ectodermal explants (animal

cap assay) to distinguish direct effects on the ectoderm from secondary effects via the mesoderm. Consistent with the in vivo observation, *Xfoxi1a* expression was diminished by *Chd* injection in the animal cap assay (Fig. 2K-M,O,P), but its expression was rescued by co-injection of *Bmp4* (Fig. 2K, lane 4), indicating that Bmp signaling positively regulates *Xfoxi1a* expression by directly acting in the ectoderm.

We next studied the role of Wnt signaling in *Xfoxi1a* expression. Microinjection of a *Wnt1*-expression plasmid (2.5 pg DNA/cell) into the animal blastomeres markedly reduced *Xfoxi1a* expression (100%, *n*=14; Fig. 2D). By contrast, overexpression of the Wnt inhibitor gene *Dkk1* (125 pg/cell) (Glinka et al., 1998) resulted in the expansion of *Xfoxi1a* expression into the posteroventral ectoderm (38%, *n*=16; Fig. 2E). Consistent with this, the animal cap assay showed that *Wnt1* suppressed *Xfoxi1a* expression in ectodermal explants (100%, *n*=30; Fig. 2L,N), without inducing *Sox2* (Fig. 2O,Q).

These findings suggest that *Xfoxi1a* expression in the gastrula ectoderm is regulated positively by Bmp signals and negatively by Wnt signals; this regulation presumably occurs as a consequence of the modifications that specify the DV (ventralization by *Bmp4*) and AP (posteriorization by Wnt) ectodermal identities. Consistent with this idea, *Xfoxi1a* expression in the neurula ectoderm (Fig. 2F) was suppressed by *Chd* (93.8%, *n*=16; Fig. 2H) and by *pCS2Wnt1* (93.8%, *n*=16; Fig. 2I), and upregulated by *Dkk1* (100%, *n*=15; Fig. 2J). In contrast to the effect on the gastrula ectoderm (Fig. 2B), injection of *pCS2Bmp4* did not cause expansion of *Xfoxi1a* in the neurula head ectoderm (*n*=16; Fig. 2G), suggesting that late *Xfoxi1a* expression requires finer local regulation in the neurula ectoderm.

Xfoxi1a is essential for the expression of non-neural ectodermal genes in the head

To understand the role of *Xfoxi1a/b* in early head ectodermal patterning, we performed loss-of-function experiments by injecting morpholino antisense oligonucleotides (MOs; Fig. 3A for the design; Fig. 3B-D for the efficiency and specificity tests using flag-tagged *Xfoxi1a/b* constructs). The unilateral injection of *Xfoxi1a-MO* into two right animal blastomeres at the eight-cell stage (12.5 ng/cell) expanded the expression of the neural plate marker *Sox2* in the anterior ectodermal region (48.3%, *n*=31; Fig. 3E), consistent with the in vivo expression pattern of *Xfoxi1a*. Conversely, the expression of the epidermal markers *XK81* (embryonic type I keratin) (Jonas et al., 1985) and *Dlx3* (Papalopulu and Kintner, 1993; Dirksen et al., 1994; Feledy et al., 1999) decreased on the injected side (40%, *n*=45 and 36.4%, *n*=44; Fig. 3F,G, respectively). *Xfoxi1a-MO* injection also inhibited the expression of markers for the cephalic intermediate ectoderm, including for the neural crest (*FoxD3*) (Sasai et al., 2001, 72.7%, *n*=11; Fig. 3H), cement gland (*Xag1*) (Sive et al., 1989) (45.7%, *n*=35; Fig. 3I) and pre-placodal region (*Six1*, 90%, *n*=22 in Fig. 3J) [*Eya1* (David et al., 2001) 63.6%, *n*=11; data not shown]. In the tailbud-stage embryo, *Six1* expression in the nasal placode disappeared on the injected side (100%, *n*=11; Fig. 3K). *Xfoxi1a-MO* injection caused no change in the expression of the axial mesodermal marker *Chd* (*n*=18; Fig. 3L) or the paraxial mesodermal marker

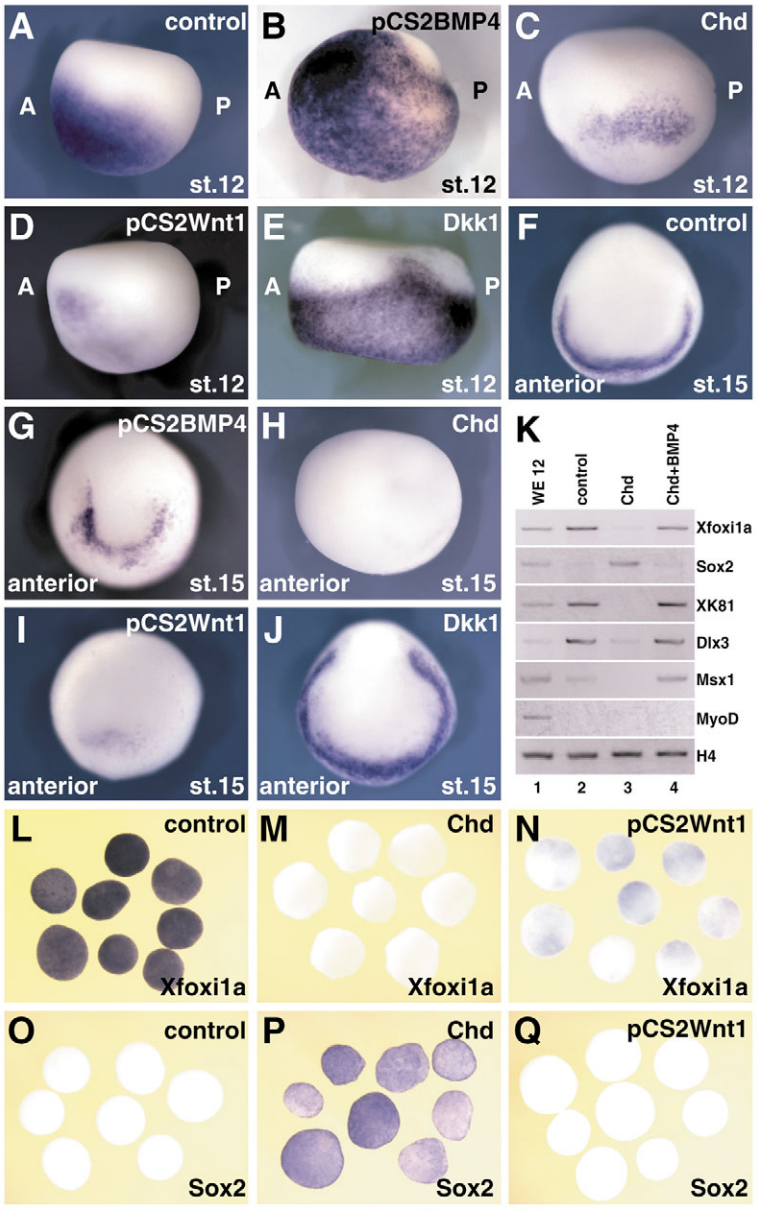

Fig. 2. Regulation of *Xfoxi1a* expression by Bmp and Wnt signals. (A-J) Effects of Bmp or Wnt signals on *Xfoxi1a* expression in the gastrula and neurula were analyzed by injecting *pCS2-BMP4* (2.5 pg DNA/cell) (B,G), *Chd* (50 pg RNA /cell) (C,H), *pCS2-Wnt1* (2.5 pg DNA/cell) (D,I) or *Dkk1* (125 pg RNA/cell) (E,J) into all the animal blastomeres of eight-cell embryos. The embryos were fixed at stage 12 or 15, then whole-mount in situ hybridization was performed with a probe for *Xfoxi1a*. Control embryos are shown in A and F. (K) Gene expression in animal caps injected with RNAs encoding Chd (200 pg) or Chd (200 pg) +Bmp4 (20 pg) was analyzed by RT-PCR. (L-Q) Animal caps given a *Chd* mRNA (200 pg; M and P) or *pCS2-Wnt1* (10 pg; N and Q) injection were excised at stage 9, and then cultured in LCMR until sibling embryos reached stage 12. The *Xfoxi1a* (L-N) or *Sox2* (O-Q) probes were used for whole-mount in situ hybridization.

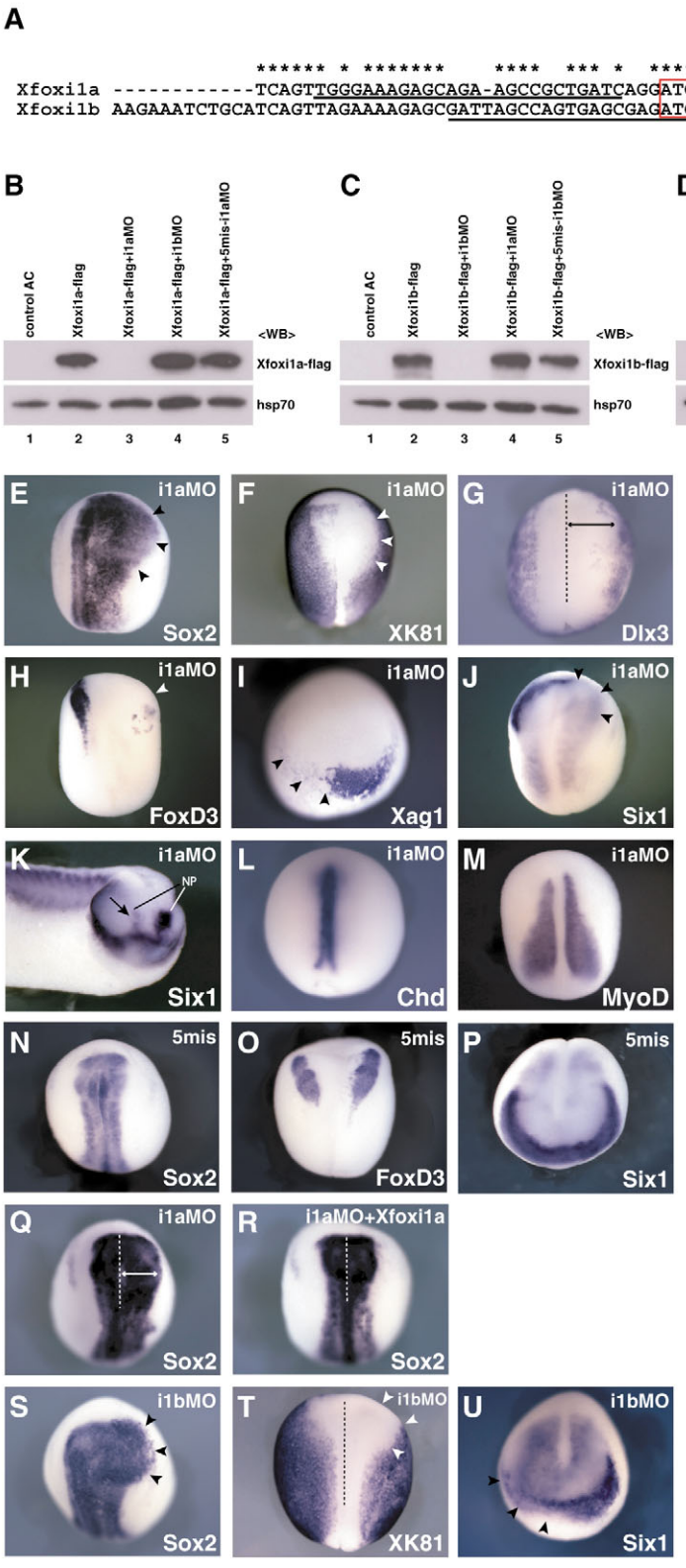

Fig. 3. Loss of *Xfoxi1a/b* function results in an expansion of the neural plate and reduction of non-neural ectodermal tissues. (A) The *Xfoxi1a/b-MO* binding sites are shown with the underlines. The red box indicates the start codon. Identical nucleotides are marked with asterisks. (B) Flag-tagged *Xfoxi1a* mRNA (50 pg/cell) was injected with *Xfoxi1a-MO* (2.5 ng/cell), *Xfoxi1b-MO* (2.5 ng/cell) or the control five-base mismatched *Xfoxi1a* MO into animal cells of eight-cell embryos. (C) Flag-tagged *Xfoxi1b* mRNA (50 pg/cell) was injected with *Xfoxi1b-MO* (2.5 ng/cell), *Xfoxi1a-MO* (2.5 ng/cell) or control five-base mismatched *Xfoxi1b* MO into animal cells of eight-cell embryos. (D) Flag-tagged Δ5′UTR-*Xfoxi1a* mRNA (50 pg/cell) was injected with *Xfoxi1a-MO* (2.5 ng/cell) or *Xfoxi1b-MO* (2.5 ng/cell) into animal cells of eight-cell embryos. Animal caps were excised at stage 9 and cultured until stage 11. Xfoxi1a-flag (B,D) or Xfoxi1b-flag (C) proteins were detected by western blot analysis using an anti-flag antibody. Hsp70 was used as the loading control. Δ5′UTR means that the synthetic mRNA contains only the coding sequence and not the target sequence of *Xfoxi1a/b-MO*. (E-U) *Xfoxi1a-MO* (12.5 ng/cell; i1aMO; E-M), five-base mismatched control MO of *Xfoxi1a* (12.5 ng/cell; 5mis; N-P), *Xfoxi1a-MO* (12.5 ng/cell) ± *Xfoxi1a* mRNA (25 pg/cell; Q,R) or *Xfoxi1b-MO* (12.5 ng/cell; i1bMO; S-U) was injected into two unilateral blastomeres of eight-cell embryos. Embryos were harvested at stage 14-15 (E-J,L-U) or stage 24 (K) and analyzed by whole-mount in situ hybridization with the probes indicated in each panel. (E-H,J,L-O,Q-T) Dorsal view (anterior towards the top); (I,K,P,U) anterior view (dorsal towards the top). Injected sides are marked with arrowheads. Dashes indicate the midline. NP, nasal placode. Double-headed arrows in G,Q show the expansion of Dlx3– and Six2+ regions, respectively.

MyoD (*n*=10; Fig. 3M), suggesting that the effects of *Xfoxi1a-MO* on ectodermal patterning are not secondary to the defect in mesodermal development. The control MO with a five-base mismatch (see Materials and methods) showed no effects on the expression of the marker genes (Fig. 3N-P). The expansion of *Sox2* expression and the repression of *FoxD3* and *Six1* caused by *Xfoxi1a-MO* injection were reversed by co-injecting wild-type *Xfoxi1a* mRNA lacking the *Xfoxi1a-MO* binding site (no expansion in 87.5%, *n*=16; and no repression in 43.5%, *n*=23, 86.9%, *n*=23, respectively; Fig. 3Q,R and data not shown). Similar observations were obtained in the knockdown experiments using *Xfoxi1b-MO* (Fig. 3S-U, and data not shown). The co-injection of *Xfoxi1a-MO* and *Xfoxi1b-MO* produced qualitatively indistinguishable effects from single injections (data not shown). These results demonstrate that a sufficient expression level of Xfoxi1a/b (higher than a certain threshold) is essential for the development of the cephalic non-neural ectoderm, and that *Xfoxi1a/b* has a pivotal role in the 'non-neural versus neural' specification of the head ectoderm.

We next performed the animal cap assay to further study the requirement of the *Xfoxi1a/b* function for the non-neural specification of the ectoderm. In RT-PCR analysis (Fig. 4), control animal caps (prepared at stage 9 and cultured until stage 14) strongly expressed the non-neural ectodermal markers *XK81*, *Msx1* and *Dlx3* (lane 2). Consistent with the in vivo study, injection of the MOs for both *Xfoxi1a* and *Xfoxi1b* (but not their corresponding five-base mismatched MOs) significantly suppressed *XK81*, *Msx1* and *Dlx3* (lanes 5 and 6),

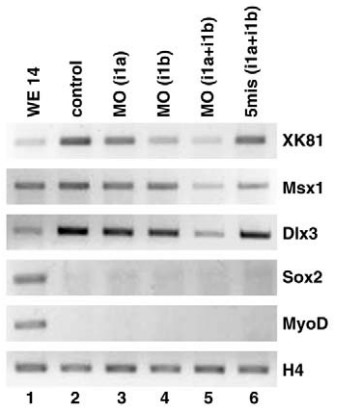

Fig. 4. Loss of *Xfoxi1a/b* function leads to reduction of epidermal tissue in naïve ectodermal cells. Animal caps given injection of *Xfoxi1a-MO* (50 ng), *Xfoxi1b-MO* (50 ng), *Xfoxi1a-MO+Xfoxi1b-MO* (25 ng each) or five-base mismatched control MOs for *Xfoxi1a* and *Xfoxi1b* (25 ng each) were excised from stage 9 embryos, and then cultured until sibling embryos reached stage 14. RT-PCR was performed using primers to detect the neural marker *Sox2*, non-neural ectodermal markers (*XK81, Dlx3, Msx1*) and the mesodermal marker *MyoD*. *H4* (histone H4) was used as the loading control.

suggesting that the *Xfoxi1a/b* function is essential for naïve ectodermal cells to differentiate into the non-neural ectodermal fate in vitro. In contrast to the in vivo situation (Fig. 3), injection of a single MO, i.e. against pseudoallele *Xfoxi1a* or *Xfoxi1b* (lanes 3 and 4; at a dose sufficient to evoke the in vivo phenotypes), caused only moderate suppression, suggesting that the extent of the sensitivity to the gene dose is somehow context-dependent in these cases. Interestingly, the neural marker *Sox2* was not substantially induced in the animal caps by MO injections in any cases (lanes 3-5).

Xfoxi1a overexpression induces epidermal differentiation in vivo and in vitro

To further understand the role of *Xfoxi1a/b*, we performed gain-of-function studies. The unilateral injection of *Xfoxi1a* mRNA (25 pg/cell) into the right animal blastomeres of eight-cell stage embryos caused a significant reduction of *Sox2* expression (50%, *n*=52; Fig. 5A) in the anterior neural plate. By contrast, ectopic expression of the epidermal markers *XK81* (33.3%, *n*=54; Fig. 5B) and *Dlx3* (39.5%, *n*=43; Fig. 5C) in the neural plate region was observed on the injected side. These gain-of-function phenotypes are consistent with the observations in the loss-of-function analysis (Fig. 3), supporting the idea that *Xfoxi1a/b* plays a decisive role in the non-neural specification of the head ectoderm.

In the animal cap assay (Fig. 5F), the co-injection of *Xfoxi1a* suppressed the *Chd*-induced *Sox2* expression (lanes 3 and 4), while the expressions of the epidermal/non-neural ectodermal markers (*XK81*, *Dlx3, Msx1* and *Xfoxi1a*), which were suppressed by *Chd*, were rescued. The mesodermal marker *MyoD* was not induced regardless of the mRNA injection. Next, we further analyzed the relationship between *Xfoxi1a* and Bmp signaling by co-injecting with the dominant-negative Bmp receptor (*dnBMPR*) (Suzuki et al., 1994). Neural differentiation caused by *dnBMPR* injection in the animal cap was suppressed by co-injecting *Xfoxi1a* (Fig. 5G). Although Bmp signaling was blocked at the receptor level, *Sox2* was suppressed by *Xfoxi1a*, while the non-neural ectodermal marker *XK81* was induced (lanes 3 and 4). These suggest that *Xfoxi1a* does not act upstream of BMPR, but rather functions downstream and/or in a parallel fashion. Taken together, these findings indicate that *Xfoxi1a* promotes epidermal differentiation at the cost of neural differentiation both in vivo and in vitro.

Xfoxi1a overexpression in the embryo suppressed the intermediate ectodermal markers *FoxD3* and *Six1* (67%, *n*=43,

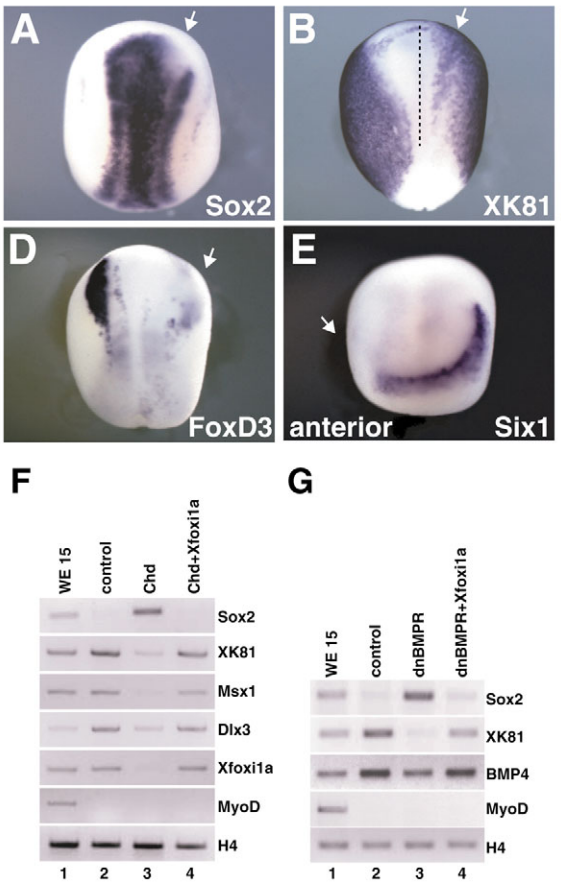

Fig. 5. Microinjection of *Xfoxi1a* mRNA induces epidermal differentiation and suppresses neural induction in both in vivo and in vitro. *Xfoxi1a* mRNA (25 pg/cell) was injected into two unilateral blastomeres of eight-cell embryos. Embryos were fixed at stage 14-15 and then whole-mount in situ hybridization was performed with the following probes. (A) *Sox2*, (B) *XK81*, (C) *Dlx3*, (D) *FoxD3* and (E) *Six1*. (A-D) Dorsal view (anterior towards top); (E) anterior view (dorsal towards the top). Dashes indicate the midline. White arrows indicate the injected side. The activity of *Xfoxi1a* (12.5 pg/cell) was assessed by RT-PCRs in *Chd* (50 pg/cell)-injected animal caps (F) or dominant-negative Bmp receptor (*dnBMPR*) (100 pg/cell)-injected animal cap (G). RNAs were injected into all the animal blastomeres of eight-cell embryos. The animal caps were excised at stage 9 and cultured until sibling embryos reached stage 15. The expression patterns of the neural marker *Sox2*, the non-neural ectodermal markers (*XK81*, *Dlx3*, *Msx1*, *Xfoxi1a*, *Bmp4*), the mesodermal marker *MyoD* were analyzed. H4 was used as the loading control.

59%, *n*=59, respectively; Fig. 5D,E). These phenotypes were similar to those with the loss-of *Xfoxi1a* function (Fig. 3H,J), suggesting the possibility that the inhibition by *Xfoxi1a* overexpression involves certain indirect effects on the specification of the intermediate ectoderm.

Xfoxi1a promotes epidermal development by acting during gastrulation

As shown in Fig. 1, the in vivo expression of *Xfoxi1a* dynamically changes during gastrulation and neurulation, suggesting the possibility that *Xfoxi1a* plays distinct roles in a stage-dependent manner. To examine the crucial period of the *Xfoxi1a* function in the 'non-neural' specification of the head ectoderm, we performed a temporally controlled misexpression experiment by using an inducible fusion protein construct comprising *Xfoxi1a* and the ligand-binding domain of the human glucocorticoid receptor (*GR-Xfoxi1a*) (Kolm and Sive, 1995). *GR-Xfoxi1a* mRNA (50 pg/cell) was injected unilaterally into the animal blastomeres of eight-cell embryos. In the absence of dexamethasone (Dex), the expressions of *Sox2, XK81, FoxD3* and *Six1* appeared normal in the injected embryos at the mid-neurula stage (Fig. 6A,D,G,J). When 10 μM Dex was added to the medium at stage 11, a significant reduction of *Sox2* was reproducibly observed on the *GR-Xfoxi1a*-injected side (53.4%, *n*=43; Fig. 6B), whereas embryos treated with Dex from stage 13 onwards exhibited no change in *Sox2* expression (*n*=13; Fig. 6C). Consistent with this finding, expansion of the epidermal maker *XK81* was seen in the neural plate of *GR-Xfoxi1a*-injected embryos treated with Dex from stage 11 onwards (35.7%, *n*=42; Fig. 6E) but not in those treated from stage 13 onwards (*n*=12; Fig. 6F). Similarly, the reduction of the neural crest and preplacodal markers *FoxD3* and *Six1* was observed in *GR-Xfoxi1a*-injected embryos treated with Dex from stage 11 onwards (55%, *n*=20; 74%, *n*=20, respectively; Fig. 6H,K), but not in those treated with Dex from stage 13 onwards (*n*=20, *n*=25, respectively; Fig. 6I,L). To eliminate the possibility that the GR-Xfoxi1a protein became degraded and ineffective by stage 13, we assessed the protein expression levels by western blot (flag-tagged *GR-Xfoxi1a*, which causes phenotypes indistinguishable from those caused by *GR-Xfoxi1a*, was used). As shown in Fig. 6M, the flag-tagged *GR-Xfoxi1a* products were detected at similar levels in the gastrula and neurula stages. Taken together, these observations suggest that *Xfoxi1a* promotes the epidermal specification by acting during the gastrula stages in vivo.

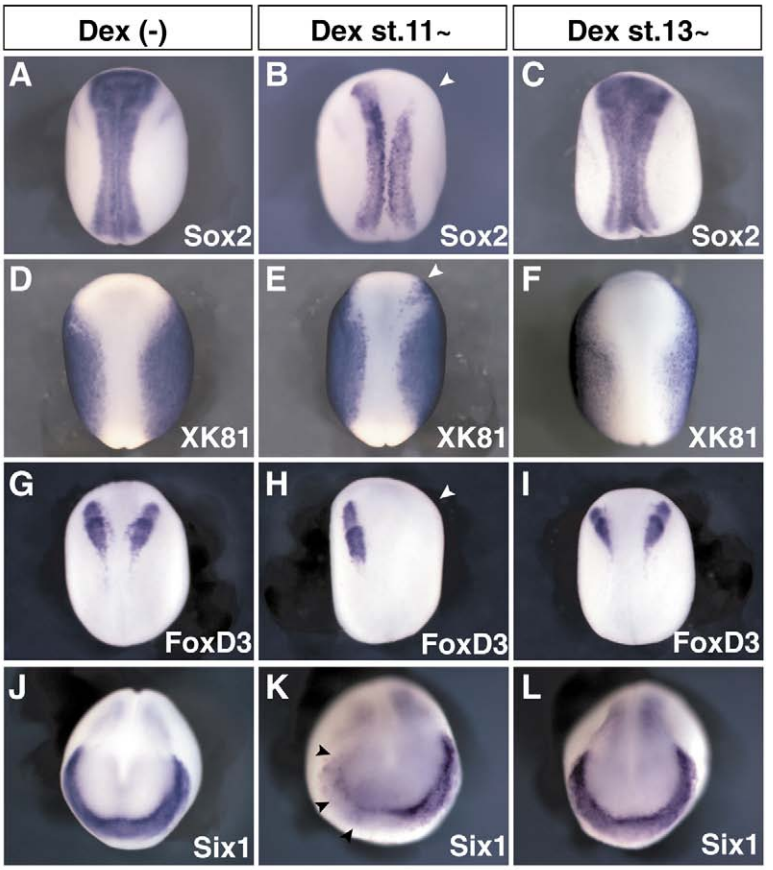

Fig. 6. Crucial time window of *Xfoxi1a*. *GR-Xfoxi1a mRNA* (50 pg/cell) was injected into two unilateral animal blastomeres of eight-cell embryos. Dex was added at stage 11 (B,E,H,K) or stage 13 (C,F,I,L). Embryos were harvested at stage 15 and used for whole-mount in situ hybridization with a probe for *Sox2* (A-C), *XK81* (D-F), *FoxD3* (G-I) or *Six1* (J-L). Embryos without Dex treatment (A,D,G,J) were used as the negative control. Arrowheads indicate the injected side. (M) Flag-tagged *GR-Xfoxi1a* mRNA (50 pg/cell) was injected into all the animal blastomeres of eight-cell embryos. Animal caps were excised at stage 9 and cultured until stage 11, 13 or 15. The intact form of flag-GR-Xfoxi1a was detected by western blot analysis. Hsp70 was used as the loading control.

Discussion

Roles of *Xfoxi1a* in the early determination of the cephalic non-neural ectoderm

In mouse and zebrafish genetic studies, *Foxi1*-related genes have been shown to play essential roles in the formation of the head ectodermal derivatives (Hulander et al., 1998; Lee et al., 2003; Nissen et al., 2003; Riley and Phillips, 2003; Solomon et al., 2003a). Mouse *Foxi1* is required for normal development of the inner ear (Hulander et al., 1998). In zebrafish, *foxi1* is the responsible gene for the *hearsay* mutant, in which the otic placode formation and jaw development are impaired (Riley and Phillips, 2003; Solomon et al., 2003a). Multiple *Foxi1*-related genes exist in each vertebrate species and can be classified into three subgroups according to their structures. Interestingly, mouse *Foxi1*, zebrafish *foxi1* and *Xfoxi1a/b/c* belong to distinct subgroups: B, A and C, respectively (Solomon et al., 2003b). At present, it is not clear whether these subgroup factors function for ectodermal patterning in a distinct or redundant manner (Ohyama and Groves, 2004).

The present work has introduced a new role for a *Foxi1* family member, *Xfoxi1a/b*, in the ventral specification of the early head ectoderm during gastrulation. During the mid- and

late gastrula stages, *Xfoxi1a/b* is expressed in the anteroventral ectoderm. This gastrula expression is complementary to that of *Sox2*, indicating that all of the head ectoderm except for the neural plate tissues expresses *Xfoxi1a* (Fig. 1). Consistently, the loss-of-function study has demonstrated that *Xfoxi1a/b* is essential for the proper development of the non-neural domain of the head ectoderm (epidermis, cement gland, neural crest and placodes) and for suppression of the ectopic expansion of the neural plate (Fig. 3). Conversely, misexpression of *Xfoxi1a* induces ectopic *keratin* expression and suppresses *Sox2* expression in the neural plate region (Fig. 5). This activity of *Xfoxi1a* is limited to the gastrula stage (Fig. 6). These results indicate that *Xfoxi1a/b* plays a pivotal role for the 'neural versus non-neural' decision of the head ectoderm during gastrulation.

In the animal cap study, overexpression of *Xfoxi1a* inhibits neural differentiation caused by the injection of *Chd* (Fig. 5D) or *dnBMPR* (Fig. 5G), demonstrating that *Xfoxi1a* can exert an anti-neuralizing activity in the isolated ectodermal tissue. In addition, as the effect of *dnBMPR* is reversed by *Xfoxi1a*, it is likely that *Xfoxi1a* does not act upstream of *Bmpr* (although *Xfoxi1a* weakly induces *Bmp4* in the animal cap; Fig. 5G, lane 4), but rather acts downstream of *Bmpr* or in parallel.

Interestingly, *Xfoxi1a/b-MO* injection (at the amount sufficient for keratin suppression and *Sox2* expansion in vivo) suppresses the epidermal markers (*XK81*, *Msx1* and *Dlx3*) but does not induce the neural marker *Sox2* in the animal cap explant (Fig. 4). This suggests the possibility that the expansion of *Sox2* expression by *Xfoxi1a/b-MO* in vivo (Fig. 3) depends on some additional factors, although *Xfoxi1a/b* regulates the epidermal fate determination in a tissue-autonomous manner. This idea is supported by our preliminary observation that the ectopic *Sox2* expression in the embryo is always limited to the lateral region of the head ectoderm and not found in the more ventral region. One candidate factor may be Fgf signals, as a recent report (Delaune et al., 2005) has shown that Fgf signaling is required for anti-Bmp factors to induce ectopic *Sox2* expression in the ventral-most part of the ectoderm.

The molecular mechanism underlying the regulation of ventral specification of the head ectoderm by *Xfoxi1a/b* remains elusive. *Dlx3* and *Msx1*, which are required for non-neural ectodermal development (Suzuki et al., 1997; Feledy et al., 1999; Beanan and Sargent, 2000; Woda et al., 2003), may be among candidate mediators of *Xfoxi1a/b* activities as their expression is positively regulated by *Xfoxi1a* (Figs 3-5 and data not shown). The exact relationship between these factors and *Xfoxi1a* should be carefully analyzed along the temporal axis by using the combination of MOs and inducible constructs in future investigation. Our preliminary study has shown that *Xfoxi1a-MO* injection (which causes the expansion of *Sox2* expression) does not significantly suppress *Bmp4* expression in the head region (data not shown). This suggests that the effect of *Xfoxi1a-MO* is not primarily mediated by the inhibition of *Bmp4* expression, consistent with the *dnBMPR* study. In future, it will be important to systematically identify downstream target genes (and possible co-factors) of *Xfoxi1a* in the ventral specification.

Roles of *Xfoxi1a/b* in the patterning of the intermediate head ectoderm

This study has mainly focused on the role of the early *Xfoxi1a/b* function in the ventral specification of the head ectoderm during gastrulation. Later, by the mid-neurula stage, *Xfoxi1a* expression fades in the ventralmost area of the head ectoderm and becomes limited to the preplacodal region (Fig. 1). Although this late expression pattern of *Xfoxi1a/b* seems relevant to the requirement of the *Foxi1* family genes for proper development of the head placodes of other species (Hulander et al., 1998; Lee et al., 2003; Nissen et al., 2003; Solomon et al., 2003a), the exact role of *Xfoxi1a/b* in late ectodermal patterning requires more careful interpretation. The intermediate head ectoderm (which gives rise to the neural crest, cement gland and preplacodal region) is complex and contains considerable heterogeneity even within the preplacodal region (Schlosser and Ahrens, 2004).

An intriguing but slightly puzzling observation regarding the role in the regulation of intermediate ectodermal genes is that the phenotypes caused by *Xfoxi1a* overexpression are basically the same as those with the loss-of *Xfoxi1a* function; both result in suppression of *FoxD3* and *Six1* (Figs 3 and 5). This is in contrast to the situation of the regulation of *Sox2* and *XK81* by *Xfoxi1a/b*, in which gain- and loss-of-function experiments show the opposite phenotypes (Figs 3 and 5). One interpretation of this discrepancy is that *Xfoxi1a* affects the development of the intermediate head ectoderm in a non-cell-autonomous fashion; both augmentation and attenuation of *Xfoxi1a* may interfere with the interactions between the neural plate and epidermis, which are required for the proper differentiation of the intermediate ectoderm (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; LaBonne and Bronner-Fraser, 1999; Glavic et al., 2004). This idea is in agreement with the largely non-overlapping expression patterns of *Xfoxi1a* and *Six1* or *FoxD3* in the mid-neurula (Fig. 1H and data not shown). Alternatively, the role of *Xfoxi1a* could be cell-autonomous, given that *Xfoxi1a* is expressed throughput the *Sox2*-negative head ectoderm (which should include the intermediate ectoderm) at the mid-gastrula stage (Fig. 1D), unlike at the mid-neurula stage (Fig. 1G). In this case, the gain- and loss-of-function phenotypes in the intermediate head ectoderm should be caused by distinct mechanisms.

The study with *GR-Xfoxi1a* suggests that the inhibitory effects of *Xfoxi1a* on the intermediate ectodermal markers are related to the *Xfoxi1a* activity before the late gastrula stage (Fig. 6). *FoxD3* and *Six1* expressions at the neurula stage are clearly suppressed when *GR-Xfoxi1a*-injected embryos are treated with Dex from stage 11 but not from stage 13 (Fig. 6G-L). However, as the neural plate marker *Sox2* is affected in a similar manner (Fig. 6A-C), it remains to be clarified whether the suppression of *FoxD3* and *Six1* is directly or indirectly caused by *Xfoxi1a*.

Regulation of early *Xfoxi1a* expression

Early *Xfoxi1a* expression in the anteroventral ectoderm (stage 12) is strongly influenced by Bmp and Wnt signals (Fig. 2). Working as upstream regulators, Bmp signaling positively controls *Xfoxi1a* expression in the ectoderm whereas Wnt signaling has a negative effect. The role of Bmp in the DV patterning of the cephalic non-neural ectoderm described here is in agreement with a previous report (Wilson et al., 1997). Although Wnt signals are known to be crucial for the AP patterning of the CNS (and of the mesoderm), experimental knowledge about their roles in the AP patterning of the non-

neural ectoderm has been limited. Both our in vivo and in vitro analyses (Fig. 2) have shown that Wnt signaling suppresses *Xfoxi1a*, indicating a direct regulatory role of Wnts in the determination of the cephalic non-neural ectoderm. A consistent effect of Wnt signals on *Xfoxi1a* expression is also found in the neurula embryo (Fig. 2I,J).

By contrast, the late *Xfoxi1a* expression at the neurula stage responds to Bmp4 in a slightly different manner. Although *Xfoxi1a* expression is also suppressed by *Chd*, injection of the Bmp-expression plasmid does not upregulate *Xfoxi1a* expression at this stage (Fig. 2G,H). This may be explained by the stage-dependent difference of the *Xfoxi1a* expression domains. In contrast to the wide expression domain in the anteroventral ectoderm at the late gastrula stage, *Xfoxi1a* expression at the mid-neurula stage is limited to a band in the head ectoderm, which is narrow in the dorsoventral direction (Fig. 1F). Therefore, it is likely that the late *Xfoxi1a* expression requires some additional positional information other than the ventralizing signal of Bmp4.

The present study suggests a role of *Xfoxi1a/b* as an important player that mediates early patterning signals (such as Bmp and Wnt) in the ventral specification of the head ectoderm. Further studies of the regulation and function of *Xfoxi1a/b* should improve our understanding of the molecular mechanisms that underlie the complex multiple-step patterning of the vertebrate head ectoderm.

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