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Regulation of *XFGF8* gene expression through SRY (sex-determining region Y)-box 2 in developing *Xenopus* embryos

Yong Hwan Kim[A,B], *Jee Yoon Shin*[A], *Wonho Na*[A], *Jungho Kim*[A], *Bong-Gun Ju*[A,C] *and Won-Sun Kim*[A,C]

[A]Department of Life Science, Sogang University, Seoul 121-742, Korea.
[B]Present address: Department of Physiology and Functional Genomics, College of Medicine, University of Florida, Gainesville, FL 352, USA.
[C]Corresponding authors. Emails: wskim@sogang.ac.kr; bgju@sogang.ac.kr

Abstract. Fibroblast growth factors (FGFs) function as mitogens and morphogens during vertebrate development. In the present study, to characterise the regulatory mechanism of *FGF8* gene expression in developing *Xenopus* embryos the upstream region of the *Xenopus FGF8* (*XFGF8*) gene was isolated. The upstream region of the *XFGF8* gene contains two putative binding sites for the SRY (sex-determining region Y)-box 2 (SOX2) transcription factor. A reporter assay with serially deleted constructs revealed that the putative SOX2-binding motif may be a critical *cis*-element for *XFGF8* gene activation in developing *Xenopus* embryos. Furthermore, *Xenopus* SOX2 (XSOX2) physically interacted with the SOX2-binding motif within the upstream region of the *XFGF8* gene *in vitro* and *in vivo*. Depletion of endogenous XSOX2 resulted in loss of *XFGF8* gene expression in midbrain–hindbrain junction, auditory placode, lens placode and forebrain in developing *Xenopus* embryos. Collectively, our results suggest that XSOX2 directly upregulates *XFGF8* gene expression in the early embryonic development of *Xenopus*.

Additional keywords: brain development, gene regulation, promoter analysis.

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Introduction

Fibroblast growth factors (FGFs), a family of secreted proteins encoded by at least 23 genes in mammals, function as mitogens and morphogens in a variety of cell types during developmental processes (Böttcher and Niehrs 2005; Itoh 2007). In particular, FGF8 has been shown to play important regulatory roles in gastrulation, limb development, midbrain–hindbrain formation and myogenesis. During chick and mouse development, *FGF8* is expressed in the primitive streak, optic placode, otic vesicle, nose placode, tail bud, surface ectoderm covering the facial primordia, limb bud (Crossley and Martin 1995; Mahmood *et al.* 1995; Vogel *et al.* 1996) and the midbrain–hindbrain junction (Joyner *et al.* 2000; Creuzet *et al.* 2005; Echevarria *et al.* 2005). In developing *Xenopus* embryos, *FGF8* is expressed in the neural fold, spinal cord, midbrain–hindbrain junction, optic placode, otic vesicle, facial primordia, tail tip, limb bud, somites, pharyngeal clefts, pronephros and heart mesoderm (Christen and Slack 1997). A recent study has revealed that although *FGF8b* functions as a mesoderm inducer, *FGF8a* acts in posterior neural patterning in *Xenopus* during early development (Fletcher *et al.* 2006).

Regulatory regions of the mouse *FGF8* gene have been identified in the intron that contains binding sites for transcription factors such as Engrailed and Pbx1 (Gemel *et al.* 1999). Retinoic acid (RA) also regulates *FGF8* gene expression (Brondani *et al.* 2002). For example, the unbound retinoic acid receptor (RAR) α homodimer may bind to proximal RA response element (RARE) and induce *FGF8b* transcription. However, the ligand-bound RARα homodimer or the RARα–retinoid X receptor (RXR) α heterodimer may interact with Direct repeat 2 (DR2) in the distal promoter and induce *FGF8a* expression. In addition, paired box gene 2a (Pax2a) has been identified as a regulator of *FGF8* gene expression in zebrafish embryos for determination of the midbrain–hindbrain boundary (Inoue *et al.* 2008). Interestingly, a unique relationship between SRY (sex-determining region Y)-box (SOX) and *FGF* has been confirmed using promoter assays in mouse and human embryonic carcinoma cell lines. For example, SOX6 forms a complex with C-terminal-binding protein 2 (CtBP2) to repress *FGF3* transcription (Murakami *et al.* 2001) and SOX2 regulates *FGF4* transcription in embryonic carcinoma cells (Yuan *et al.* 1995).

The SOX proteins constitute a large family of transcription factors that have a high mobility group (HMG) DNA-binding domain with high homology and recognise the '$^{A}/_{T}/_{T}$CAA$^{A}/_{T}$G' conserved sequence motif (Wilson and Koopman 2002). It is well known that SOX transcription factors play important roles

in cell lineage determination and differentiation during vertebrate embryogenesis, either activating or repressing specific target genes through interactions with different partner proteins (Pevny and Placzek 2005; Kiefer 2007).

In the present study, we cloned the upstream regulatory sequences of the *Xenopus FGF8* (*XFGF8*) gene to investigate mechanisms regulating *XFGF8* gene expression. The results indicate that the *Xenopus* SOX2 (XSOX2) transcription factor activates gene expression via direct interaction with the upstream SOX2-binding motif of the *XFGF8* gene in early developing *Xenopus* embryos.

Materials and methods

Xenopus *embryo manipulations*

Xenopus laevis embryos were obtained by mating adult female and male *X. laevis* primed with 550 and 300 units human chorionic gonadotropin (Sigma, St Louis, MO, USA), respectively. Fertilised eggs were dejellied in 2.0% L-cysteine (Sigma), dissolved in 1× Marc's modified Ringer (composition (in mM): NaCl 110; KCl 2, $MgCl_2$ 1; $CaCl_2$ 2; $NaHCO_3$ 2; HEPES 5, pH 7.8) and cultured in 0.1× Marc's modified Ringer. Embryos were staged according to Nieuwkoop and Faber (1967).

Cloning of the upstream region of the XFGF8 *gene*

To investigate the mechanism regulating *XFGF8* gene expression, we first screened the *Xenopus* genomic DNA library (Stratagene, La Jolla, CA, USA) with a 710-bp, [α-^{32}P]-dCTP-labelled, 5′-untranslated region (UTR) of *XFGF8b* as a probe (Shim *et al.* 2005). After high stringent screening of 1.5 million lamda plaques, a 2.4-kb genomic fragment of the *XFGF8* gene was subcloned into the *Not*I site of pBluescript SK(–) plasmid (Stratagene).

Reporter plasmids and luciferase reporter assay

To determine the elements crucial for *XFGF8* regulation, a 2.4-kb genomic fragment containing 2005 bp of a putative transcriptional regulatory region and 417 bp of a 5′-UTR of the *XFGF8* gene was subcloned into the *Sac*I site of pGL2 basic luciferase reporter vector (Promega, Madison, WI, USA) and designated as −2715/Luc (nucleotides (nt) −2715 to −293; note, nt are numbered in the 5′ to 3′ direction from the translation initiation site). In addition, four deletion reporter constructs of the upstream region were generated by polymerase chain reaction (PCR) and designated as −2591/Luc, −2502/Luc, −2428/Luc and −2309/Luc. The PCR primers used to generate the deletion constructs were as follows: for −2591/Luc, 5′-AAC TGT GTC ACT GAA AGG CTC TCC C-3′ (forward) and 5′-GAG CTC ACG CCT CTC AAG AGC AAG A-3′ (reverse); for −2502/Luc, 5′-AAC TGA CCT TAT TCT CTC TCT CTC T-3′ (forward) and 5′-GAG CTC ACG CCT CTC AAG AGC AAG A-3′ (reverse); for −2428/Luc, 5′-AGA ATT TCC CTT AAT TCC ACT GTG C-3′ (forward) and 5′-GAG CTC ACG CCT CTC AAG AGC AAG A-3′ (reverse); and for −2309/Luc, 5′-CAG TGA CAA ATT TTA TGG GTC TTA T-3′ (forward) and 5′-GAG CTC ACG CCT CTC AAG AGC AAG A-3′ (reverse).

The *XFGF8* reporter constructs (50 pg) were coinjected with the pCMV-renilla luciferase internal control plasmid (5 pg) into each *Xenopus* blastomere at the 2-cell stage. Embryos were grown until the neurula stage (Stage 15). Five pools of two embryos (total 10 embryos) were assayed independently for luciferase activity and the same experiments were repeated three times using the Dual-Luciferase Assay System (Promega). Briefly, embryos were homogenised in 200 μL passive lysis buffer and 20 μL cleared lysate was assayed sequentially for firefly and renilla luciferase activities. Renilla luciferase activity served as an internal control against which the firefly luciferase activity of the *XFGF8* upstream region was normalised.

Microinjection of Xenopus *embryos*

Fertilised eggs were collected and microinjected with the reporter DNA, cDNA, synthetic RNA or morphlolinos (MO) as described previously (Sive *et al.* 2000). Microinjection was performed in 1× Marc's modified Ringer containing 3% Ficoll-400 using a Nanojector (Drummond, Broomall, PA, USA). A microelectrode puller (PP830; Narishige, Tokyo, Japan) was used to pull the needle (7.0-nL micropipettes; WPI, Sarasota, FL,USA). The diameter of the tip of the injection needle was 10–20 μm. The needle tip was clipped with forceps before use. After injection, embryos were maintained in 0.33× Marc's modified Ringer with 50 μL mL^{-1} gentamycin.

Prediction of transcription factor binding sites

Putative transcription factor binding sites were analysed using the Alggen PROMO Internet-based transcription factor binding site program (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB = TF_8.3, accessed 3 September 2009) with the default setting of 15% dissimilarity value (Messeguer *et al.* 2002; Farre *et al.* 2003).

RNA isolation and reverse transcription–PCR analysis

Total RNA was isolated from 10 embryos at various stages using TRIzol (Invitrogen, Carlsbad, CA, USA). The cDNA templates were synthesised using M-MLV reverse transcriptase (New England Biolabs, Ispwich, MA, USA). Each cDNA was amplified using *pyrobest* DNA polymerase (TaKaRa, Dalian, China). The thermal cycling conditions consisted of 57°C for 30 s, followed by an initial denaturation step at 96°C for 5 min and then 30 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 30 s. A 5-μL aliquot of each PCR product was resolved by 1% agarose gel electrophoresis and observed with ethidium bromide. Transcript levels were normalised against *Xenopus ornithine decarboxylase* (*XeODC*). The reverse transcription–PCR (RT-PCR) primers used in the present study were as follows: for *XFGF8*, 5′-GCG GAT GAA ATG TAC GGA CT-3′ (forward) and 5′-ATG CAG TTG CAC GTC TTG AG-3′ (reverse); and for *XSOX2*, 5′-GAG GAT GGA CAC TTA TGC CCA C-3′ (forward) and 5′-GGA CAT GCT GTA GGT AGG CGA-3′ (reverse). Primers for *XeODC* were as described by Agius *et al.* (2000).

Preparation of the XSOX2 *construct*

In order to obtain full-length *XSOX2* cDNA for *in vitro* transcription and electrophoretic mobility shift assay (EMSA),

cDNA was amplified using total RNA from the head region of *Xenopus* tadpoles at Stage 35. Primers were designed using the *XSOX2* cDNA sequence in GenBank (Accession no. AF005476). The PCR primers used to clone the *XSOX2* cDNA were 5′-AGG CCT CGA GAC ACG CCG CCT CGA TGT ACA G-3′ (forward) and 5′-GGC TCT AGA TTT TTC ACA TGT GCG ACA GAG-3′ (reverse). The PCR-generated *XSOX2* product was inserted into pcDNA3-Flag tag vector and pTrcHis A vector (Invitrogen).

In vitro *transcription for capped* XSOX2 *mRNA synthesis*

C-Terminal Flag octapeptide (DYKDDDDK)-tagged *XSOX2* mRNA (*XSOX2-Flag* mRNA) was synthesised using the mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA) after linearisation of pcDNA3 vector containing XSOX2-Flag by *Xba*I for overexpression of XOX2-Flag protein in *Xenopus* embryos. The mRNA synthesised automatically contains a 7-methyl guanosine cap structure at the 5′ end, as designed by the kit. The mRNA synthesised was purified by standard phenol–chloroform extraction (Sambrook and Russell 2001). Ethanol precipitation was performed at −70°C overnight, followed by centrifugation at 4°C for 30 min at 12 000*g*. Capped mRNA was resuspended in nuclease-free water.

Depletion of endogenous XSOX2 by MO

The *XSOX2* antisense MO (*XSOX2*-AS MO; 5′-AGC TCG GTC TCC ATC ATG CTG TAC-3′) was designed to deplete XSOX2 protein in developing *Xenopus* embryos (Gene Tools, Philamath, OR, USA). A random sequence standard morpholino (StdMO) and a 5′-mismatched *XSOX2* MO (*XSOX2* 5′-misMO) served as controls. Morpholinos (20 ng) were injected into each *Xenopus* blastomere at the 2-cell stage. To determine whether XSOX2-AS MO specifically reduces XSOX2 protein levels, 20 ng of each MO was coinjected with 100 pg *XSOX2-Flag* mRNA into 20 embryos at the 2-cell stage. Embryos were harvested at the mid-gastrula stage (Stage 11) and embryos were lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.25% Na-deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 50 mM TRIS-HCl, pH 7.4). Western blot analysis was performed using anti-Flag antibody (Sigma; 1 : 50 000 dilution) and anti-actin antibody (Sigma; 1 : 5000 dilution).

Expression and purification of XSOX2 protein

To test the interaction between the XSOX2 protein and the *XFGF8* genomic fragment containing the SOX2-binding motif *in vitro*, XSOX2 proteins were purified using Ni-NTA agarose (Qiagen, Valencia, CA, USA) after isopropylthio-β-D-galactoside (IPTG)-induction of *Escherichia coli* BL21 strain, which was transformed with 6× histidine-tagged *XSOX* cDNA. Then, 1 mL overnight-grown culture was added to 50 mL LB medium and incubated with shaking at 37°C until the optical density at 600 nm reached 0.6. The IPTG was then added to a final concentration of 1 mM and bacteria were cultured for additional 4 h at 37°C. Bacterial cells were harvested and washed with 0.1 M phosphate-buffered saline (PBS; pH 7.4). Bacterial lysates were obtained by sonication in 0.1 M PBS and then recentrifuged at 12 000*g* at 4°C for 15 min. The resulting supernatant containing 6× histidine-tagged XSOX2 protein was incubated with the 50% Ni-NTA agarose slurry in a column at 4°C for 60 min. The column was washed with 4 mL wash buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole (pH 8.0). Histidine-tagged XSOX2 proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0).

Electrophoretic mobility shift assay

The EMSA was performed to investigate interactions between the XSOX2 protein and the *XFGF8* genomic fragment containing the SOX2-binding motif. Double-stranded oligonucleotides were prepared by heating equal molar amounts of complementary oligonucleotides to 95°C and then cooling them to room temperature. The resulting double-stranded fragments were labelled with [α-^{32}P]-dCTP using Klenow fragment DNA polymerase (Takara). The oligonucleotide sequences used in EMSA were as follows: for wild-type SOX2-binding motif, 5′-TTG ACC AGT T**TA TTG T** TT AGT TTT CAA A-3′ (bold letters represent the putative SOX2-binding motif); and for the mutant SOX2-binding motif, 5′-TTG ACC AGT Tac cgc ggT AGT TTT CAA A-3′ (lowercase letters denote substitutions). Labelled oligonucleotides were incubated with the 6× histidine-tagged XSOX2 protein for 20 min on ice in 12 μL reaction mixture containing 10 mM HEPES (pH 8.0), 5% glycerol, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.1 mM EDTA and 2.4 μg bovine serum albumin. Electrophoresis was performed on an 8% native gel. Excess amounts of unlabelled SOX2 wild-type or mutant oligonucleotides were used for a competition assay.

Chromatin immunoprecipitation assay

To confirm endogenous interactions between the XSOX2 protein and the *XFGF8* genomic fragment containing the SOX2-binding motif, a chromatin immunoprecipitation (ChIP) assay was performed as described previously (Kim *et al.* 2004), with minor modifications. Briefly, after injection of 500 pg *XSOX2-Flag* mRNA into embryos at the 2-cell stage, the embryos were maintained until they had reached the gastrula stage. Fifty embryos were fixed in 1% formaldehyde for 1 h at room temperature and fixation was stopped by the addition of glycine to a final concentration of 125 mM for 10 min. Embryos were washed in PBS and homogenised in 600 μL lysis buffer (1% NP-40, 0.25% Na-deoxycholate, 50 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.5 mM DTT, 5 mM sodium butyrate, 50 mM TRIS-HCl, pH7.5) containing Complete Protease Inhibitor (Roche, San Francisco, CA, USA). An approximate 500-bp DNA fragment was obtained by sonication, precleared with 25 μL protein A agarose beads (Sigma) and immunoprecipitated with anti-Flag M2 (Sigma), anti-Myc 9E10 (Roche) and control mouse IgG (Sigma) at 4°C overnight. Antibody-coupled chromatins were incubated with 25 μL protein A agarose beads for 2 h at 4°C. The agarose beads were sequentially washed with Solution I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM TRIS-HCl, pH 8.0), Solution II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM TRIS-HCl, pH 8.0), Solution III (0.25 M LiCl, 1% NP-40, 1 mM EDTA, 10 mM TRIS-HCl, pH 8.0) and Tris-EDTA (TE) buffer for 5 min each time. After reverse

cross-linking at 65°C for 4 h, the bound DNA fragments were extracted by phenol–chloroform and purified using a Qiagen column. The following PCR primers were used to amplify the region containing the SOX2-binding motif (nt −2814 to −2432) in the upstream region of the *XFGF8* gene: upstream (U), 5′-ACC ATA AAA CAG CTG GCA CA-3′; and downstream (D), 5′-CTG GAA GTG TGA AGC CCA TT-3′. The thermal cycling conditions consisted of 58°C for 30 s, followed by an initial denaturation step at 96°C for 10 min and then 35 cycles of 96°C for 30 s, 57°C for 30 s and 72°C for 30 s. Finally, 5 μL of each PCR product was resolved by 1% agarose gel electrophoresis and observed with ethidium bromide.

Whole-mount in situ *hybridisation*

To examine the spatial expression of endogenous *XFGF8* in XSOX2-depleted embryos, whole-mount *in situ* hybridisation was performed (Sambrook and Russell 2001). More than 10 embryos in each experimental group were fixed in MEMFA buffer (2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde, 0.1 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS), pH 7.4). Fixed embryos were subjected to dehydration and rehydration with ascending and descending series of ethanol (25%, 50%, 75% and 100%) in 0.1% Tween-20–PBS (PBT). Embryos were subjected to prehybridisation treatment in hybridisation solution (50% formamide, 5× standard saline citrate (SSC), 1% SDS, 50 μg mL^{-1} yeast tRNA, 50 μg mL^{-1} heparin) without riboprobe at 62°C for 2 h. Hybridisation was performed in new hybridisation solution with riboprobe at 65°C overnight. Embryos were washed three times with Solution A (50% formamide, 4× SSC, 1% SDS) at 62°C for 30 min each time, followed by three washes with Solution B (50% formamide, 2× SSC) at 60°C for 30 min each time and a final three washes with TBST (140 mM NaCl, 2.7 mM KCl, 1% Tween-20, 2 mM levamisole, 25 mM TRIS-HCl, pH 7.5) at room temperature for 30 min. After preblocking of embryos with 10% heat-inactivated sheep serum in TBST for 3 h, anti-digoxigenin (DIG) fab fragment antibody conjugated to alkaline phosphatase (Roche) were used to detect DIG-labelled riboprobe at 4°C overnight. The colour reaction proceeded with BM Purple (Roche) after 10 washes with TBST for 30 min each time. The DIG-labelled antisense *XFGF8* riboprobe was transcribed by T3 RNA polymerase (Roche) from the *Xho*I-linearised pBluescript SK(–) plasmid containing *XFGF8* cDNA using DIG RNA labelling mix (Roche). After whole-mount *in situ* hybridisation, embryos were classified according to the level of *XFGF8* mRNA expression. The same experiments were repeated three times independently.

Statistical analysis

Data are presented as the mean ± s.e.m. and differences between groups were evaluated by unpaired *t*-test using Excel (Microsoft, Redmond, WA, USA). $P < 0.05$ was considered significant (Kurayoshi *et al.* 2007).

Results

Structure of the upstream region of the XFGF8 *gene*

To explore the molecular mechanisms regulating *Xenopus FGF8* gene expression in early *Xenopus* development, we screened the *X. laevis* genomic DNA library using a 5′-UTR fragment of *XFGF8b* cDNA as a probe. As a result, a clone containing a 2.4-kb upstream region of the *XFGF8* gene was obtained and its DNA sequence was analysed. The *XFGF8* genomic fragment contained 2005 bp of a putative transcriptional regulatory region and 417 bp of a partial 5′-UTR (see Fig. S1 available as an Accessory Publication to this paper). Previously, it was reported that promoters of human and mouse *FGF8* genes are TATA-less (Gemel *et al.* 1999; Brondani *et al.* 2002). We also found that the *Xenopus FGF8* gene lacks any sequence homology with a canonical TATA-box. Instead, an initiation element (Inr) consensus motif, namely YYA+1NT/AYY (where A+1 denotes the transcriptional start site), was found approximately 710 bp upstream of the translational start site in the *XFGF8* gene (Smale 1997).

Analysis of the upstream region of the XFGF8 *gene*

We first subcloned the 2.4-kb upstream region of the *XFGF8* gene into pGL2 basic luciferase vector and designated the construct −2715/Luc. As shown in Fig. 1*a*, following its injection into embryos, −2715/Luc showed strong promoter activity in developing *Xenopus* embryos compared with empty pGL2 basic luciferase vector. In fact, the activity of −2715/Luc was approximately 80% that of strong CMV promoter activity, indicating that the developing *Xenopus* embryo is a suitable system for the reporter assay and that the 2.4-kb upstream region of the *XFGF8* gene may have critical *cis*-elements for *XFGF8* gene activation. To analyse the critical regulatory region, reporter constructs containing various lengths of the upstream region of the *XFGF8* gene were generated. Interestingly, reporter activities of −2591/Luc, −2502/Luc, −2428/Luc and −2309/Luc were markedly reduced (Fig. 1*b*). These results suggest that the region between nt −2715 and −2591 contains a critical regulatory element for the transcriptional activation of the *XFGF8* gene in developing *Xenopus* embryos.

Regulation of XFGF8 *expression by XSOX2*

Analysis of putative regulatory sequences using Alggen PROMO Internet-based program for the transcription factor binding site revealed the presence of two putative SOX2 transcription factor-binding motifs in the 124-bp upstream region (nt −2715 to −2591) of the *XFGF8* gene (Fig. 2). We tested the possibility that the SOX2-binding motif may be crucial for *XFGF8* gene activation because the spatial and temporal expression patterns of *XSOX2* and *XFGF8* partially overlap in early *Xenopus* development (Fig. 3*a*; Christen and Slack 1997; Mizuseki *et al.* 1998; Wegner 1999; Hardcastle *et al.* 2000; Bylund *et al.* 2003; Nitta *et al.* 2006). To determine whether XSOX2 is required for *XFGF8* gene activation, we performed loss-of-function experiments using MOs. The specificity of the MOs was evaluated using western blot analysis after coinjection of *XSOX2-Flag* mRNA and the MOs into *Xenopus* embryos (Fig. 3*b*). Although the control MOs, such as StdMO and *XSOX2* 5′-misMO, did not significantly affect XSOX2-Flag expression, the *XSOX2*-AS MO resulted in a marked reduction in XSOX2-Flag protein levels (Fig. 3*b*). To test whether XSOX2 protein is involved in *XFGF8* gene activation, −2715/Luc was coinjected

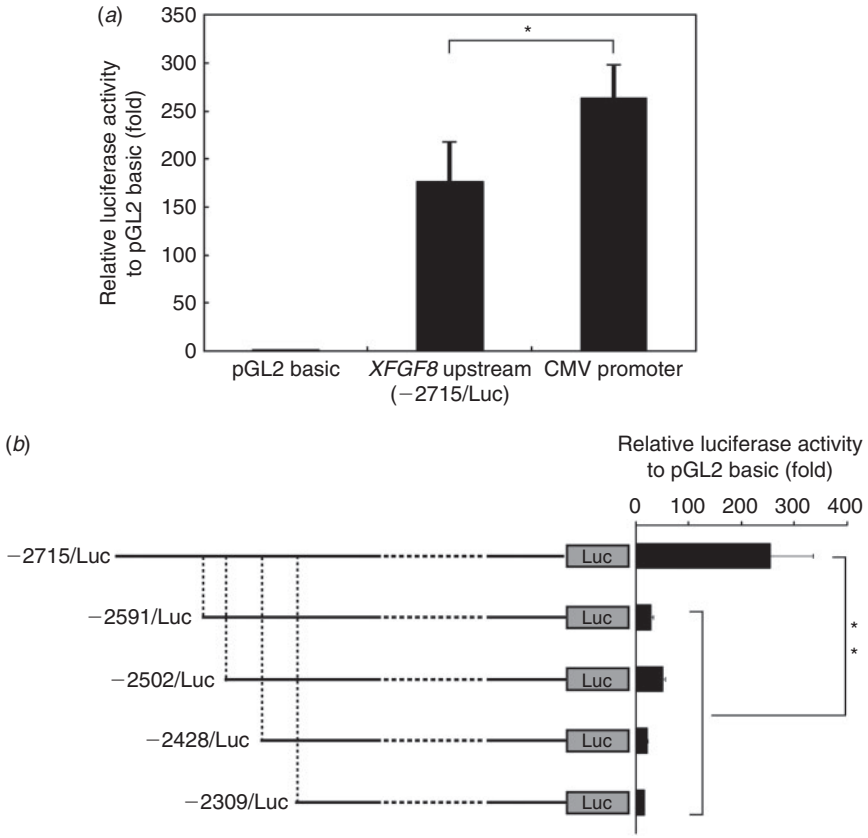

Fig. 1. Identification of *cis*-elements in the upstream region of the *Xenopus* fibroblast growth factor 8 (*XFGF8*) gene. (*a*) Luciferase reporter activity of the *XFGF8* gene construct containing a 2.4-kb long upstream region (−2715/Luc) was compared with that of the control reporter containing the CMV promoter in developing *Xenopus* embryos. (*b*) Decreased luciferase activity in the *XFGF8* reporter in which 124 bp of the *XFGF8* upstream region (nucleotides −2715 to −2591) was deleted. Data are the mean ± s.e.m. $*P < 0.05$, $**P < 0.01$.

−2715 AGCTCACAGCTCTATTTTATTCATTGACCAGT
TTATTGTTTAGTTTTCAAAGTCAAAACCTAAT
CCAATTAATCCGTCTCCCACATTTCCATAGAA
CCAAGCAGCCGATTGTCCCAATACACA −2591

Fig. 2. Nucleotide sequence of the deleted 124-bp region of the *XFGF8* gene (nucleotides −2715 to −2591). Core SRY (sex-determining region Y)-box 2 (SOX2) transcription factor binding sites are underlined.

into each blastomere with 20 ng *XSOX2*-antisense or control MOs. As shown in Fig. 3*c*, depletion of XSOX2 resulted in a significant decrease in reporter activity compared with control experiments (StdMo and *XSOX2* 5′-misMO), suggesting that XSOX2 upregulates *XFGF8* gene expression in developing *Xenopus* embryos.

Direct interaction between XSOX2 protein and the SOX2-binding motif in the XFGF8 *gene*

To further test the physical interaction between XSOX2 protein and the putative SOX2-binding motif within the upstream region of the *XFGF8* gene, we selected one of the putative SOX2-binding motifs that showed the highest similarity with the consensus SOX2-binding motif (Fig. 2). A series of EMSA was performed using the wild-type SOX2-binding motif (nt −2691 to −2663) or the mutant oligonucleotide. The XSOX2 protein formed a complex with the ^{32}P-labelled wild-type SOX2-binding oligonucleotides (Fig. 4*a*). To determine binding specificity, a competition assay was performed using a 30- or 300-fold excess molar concentration of the unlabelled oligonucleotides. The excess unlabelled wild-type SOX2-binding oligonucleotides clearly inhibited the formation of the XSOX2–DNA complex (Fig. 4*a*). However, the excess unlabelled mutant SOX2-binding oligonucleotide only marginally reduced XSOX2–DNA complex formation (Fig. 4*b*). Furthermore, the XSOX2–DNA complex was completed absent when the EMSA was performed using ^{32}P-labelled mutant SOX2-binding oligonucleotides, indicating that XSOX2 directly and specifically interacts with the upstream region of the *XFGF8* gene via the SOX2-binding motif *in vitro* (Fig. 4*c*).

We next investigated whether XSOX2 occupies the upstream region of the *XFGF8* gene *in vivo* using the ChIP assay. Chromatin from *XSOX2-Flag* mRNA-injected *Xenopus*

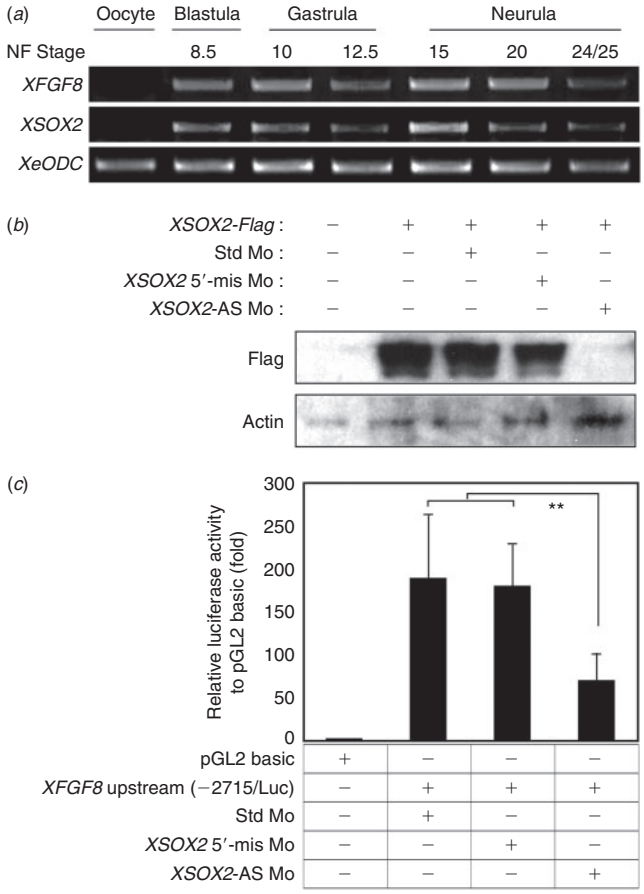

Fig. 3. Regulation of *Xenopus* fibroblast growth factor 8 (*XFGF8*) gene expression by *Xenopus* SRY (sex-determining region Y)-box 2 (XSOX2). (*a*) Temporally overlapping expression patterns of *XFGF8* and *XSOX2* in developing *Xenopus* embryos. The *Xenopus ornithine decarboxylase* (*XeODC*) transcript was amplified as a control. (*b*) Specificity of the *XSOX2* MO was examined by western blot analysis using developing *Xenopus* embryos. StdMO, random sequence standard morpholino; *XSOX2* 5′-misMO, 5′-mismatched *XSOX2* MO; *XSOX2*-AS MO, *XSOX2* antisense MO. (*c*) Significantly decreased luciferase activity of the *XFGF8* reporter gene (−2715/Luc) was observed in *XSOX2*-AS MO-injected *Xenopus* embryos. Data are the mean ± s.e.m. $^{**}P < 0.01$ NF stage, Nieuwkoop and Faber stage.

embryos was immunoprecipitated with the anti-Flag antibody. As shown in Fig. 4*d*, XSOX2 binds to the upstream region of the *XFGF8* gene that contains the SOX2-binding motifs. When normal mouse IgG and anti-Myc antibodies were used as controls, weak PCR amplification was observed within any region tested (Fig. 4*d* and data not shown). These results further indicate that XSOX2 upregulates *XFGF8* gene expression via a direct interaction with the SOX2-binding motif during early *Xenopus* development.

Role of XSOX2 in Xenopus *development*

We next examined the expression of endogenous *XFGF8* in XSOX2-depleted embryos using whole-mount *in situ* hybridisation. The *XSOX2*-AS or control MO (20 ng) were injected into one of two blastomeres at the 2-cell stage. Expression of *XFGF8* on the injected side significantly decreased, particularly in the domains where both *XFGF8* and *XSOX2* were expressed, including the midbrain–hindbrain junction, auditory placode, lens placode and forebrain in the *XSOX2*-AS MO-injected blastomere (Fig. 5*a*). However, the expression of *XFGF8* was normal or negligibly changed in embryos injected with StdMO or *XSOX2* 5′-misMO (Fig. 5*a*). In fact, the whole-mount *in situ* hybridisation results revealed that 97% of *XSOX2*-AS MO-injected embryos exhibited significantly decreased *XFGF8* mRNA expression in the midbrain–hindbrain junction, auditory placode, lens placode and forebrain (Fig. 5*b*). Collectively, these results further suggest that XSOX2 is an important transcription factor for the development of the eyes, ear and brain via direct upregulation of *XFGF8* gene expression in early developing *Xenopus* embryos.

Discussion

In developing *Xenopus* embryos, *FGF8* is expressed in the neural fold, spinal cord, midbrain–hindbrain junction, optic placode, otic vesicle, facial primordia, tail tip, limb bud, somites, pharyngeal clefts, pronephros and heart mesoderm (Christen and Slack 1997; Fletcher *et al.* 2006). Based on its unique temporal and spatial expression, it has been proposed that *XFGF8* is important for the induction and patterning of the embryo during gastrulation, limb development, midbrain–hindbrain formation and myogenesis.

In the present study, we identified XSOX2 as a transcriptional activator of the *XFGF8* gene in developing *Xenopus* embryos. Previously, it had been reported that the expression pattern and function of members of the SOX family, such as the SOXB1 and the SOXE, are similar to those of *FGF8* for the induction of neuronal differentiation (Christen and Slack 1997; Mizuseki *et al.* 1998; Wegner 1999; Hardcastle *et al.* 2000; Bylund *et al.* 2003; Nitta *et al.* 2006). Whereas the SOXB1 subgroup is essential for eye development and neurogenesis (Bylund *et al.* 2003), the SOXE group plays a crucial role in neural crest development (Taylor and Labonne 2005). Because *XFGF8* is not expressed in neural crest cells, it is reasonable to assume that SOXB1 group proteins may function as potential transcriptional regulators of *XFGF8* gene expression. Among the SOXB1 group, *SOX2* is coexpressed with *FGF8* in the early neuroectoderm and developing lens of many vertebrate embryos (Christen and Slack 1997; Mizuseki *et al.* 1998; Wegner 1999; Hardcastle *et al.* 2000; Bylund *et al.* 2003; Nitta *et al.* 2006).

Our data clearly suggest that XSOX2 interacts with the SOX2-binding motif within the upstream region of the *XFGF8* gene. In addition, expression of *XFGF8* was significantly decreased in XSOX2-depleted *Xenopus* embryos, particularly in the regions where *XFGF8* and *XSOX2* expression overlap, such as the auditory placode, lens placode and forebrain. It has been reported that SOX2 is involved in mammalian eye and forebrain development. For example, SOX2 plays a role in the development of the pituitary, forebrain and eye by inhibiting β-catenin-driven gene expression (Kelberman *et al.* 2008). Furthermore, SOX2-deficient mouse embryos exhibit abnormalities in neuronal organs, including the brain and inner ear

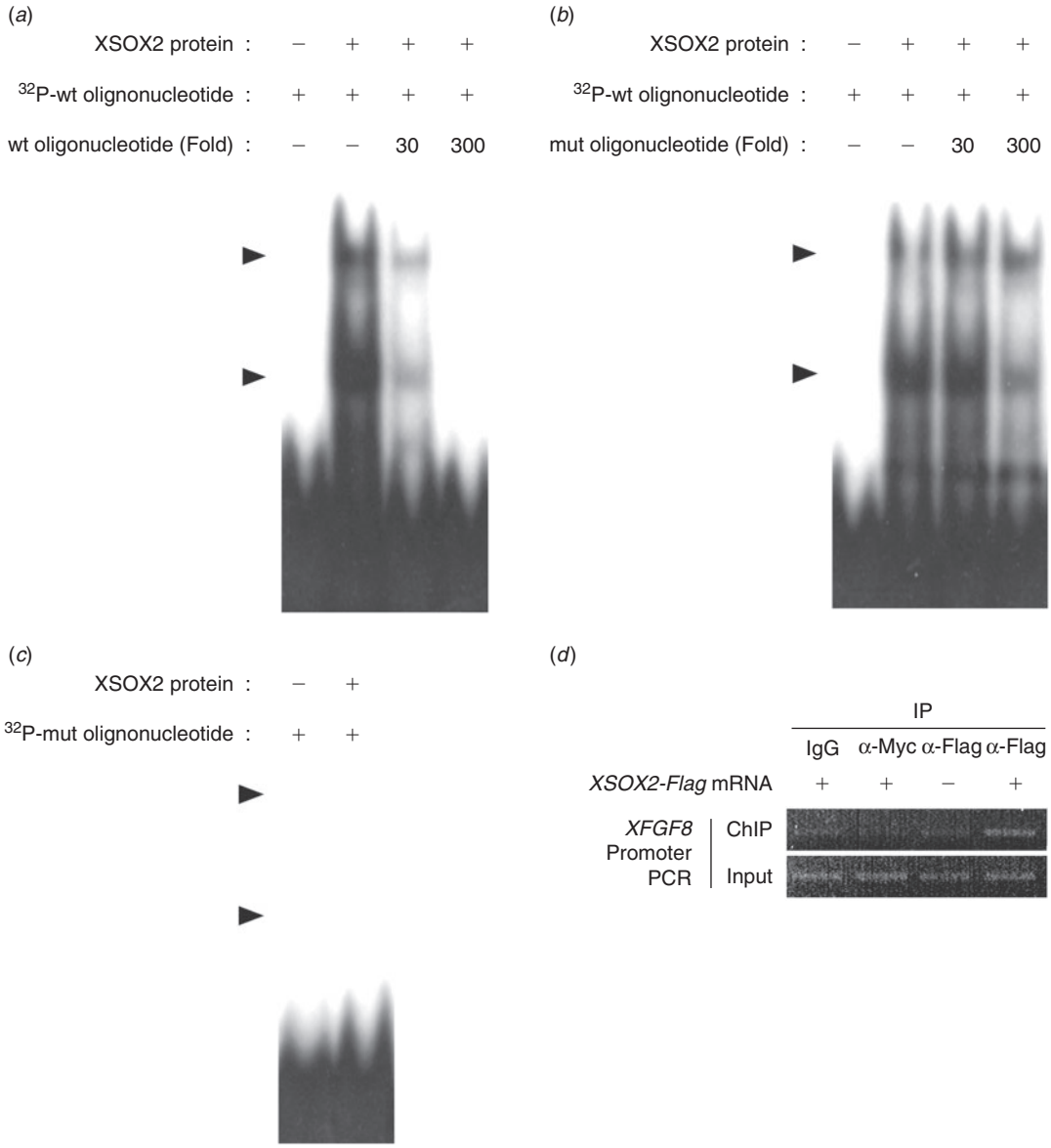

Fig. 4. Direct interaction between the putative SRY (sex-determining region Y)-box 2 (SOX2)-binding motif of the *Xenopus* fibroblast growth factor 8 (*XFGF8*) gene and *Xenopus* SOX2 (XSOX2) protein. (*a*) An electrophoretic mobility shift assay showed XSOX2 protein bound strongly to the ^{32}P-labelled SOX2-binding wild-type (wt) oligonucleotide. However, XSOX2 protein–DNA complex formation (arrowhead) was decreased in the presence of excess unlabelled wt oligonucleotide. (*b*) Excess unlabelled SOX2-binding mutant (mut) oligonucleotide marginally decreased XSOX2 protein–DNA complex formation (arrowhead). (*c*) The XSOX2 protein did not interact with the ^{32}P-labelled SOX2-binding mutant (mut) oligonucleotide. (*d*) The XSOX2 protein binds to the upstream region of the *XFGF8* gene *in vivo* in developing *Xenopus* embryos. Soluble chromatin from the *XSOX2-Flag* mRNA-injected embryos was immunoprecipitated with the anti-Flag antibody. The upstream region of the *XFGF8* gene that contains the SOX2-binding motif was amplified by polymerase chain reaction.

(Miyagi *et al.* 2008; Puligilla *et al.* 2010). Abnormal eye and brain development has also been observed in SOX2 or FGF8 MO-injected zebrafish embryos (Pujic *et al.* 2006; Okuda *et al.* 2010). These reports further support our findings that decreased *XFGF8* expression may be responsible for abnormal ear, eye and forebrain development in XSOX2-depleted *Xenopus* embryos.

However, we cannot exclude the possibilities that *XFGF8* expression is also regulated by other factors. It has been demonstrated that Engrailed, Pbx1, RARs and Pax2a play an important role in the regulation of *FGF8* expression (Gemel *et al.* 1999; Brondani *et al.* 2002; Inoue *et al.* 2008). In fact, the spatial expression of the 2.4-kb upstream region of the *XFGF8* gene-driven luciferase (−2715/Luc) was much broader than that

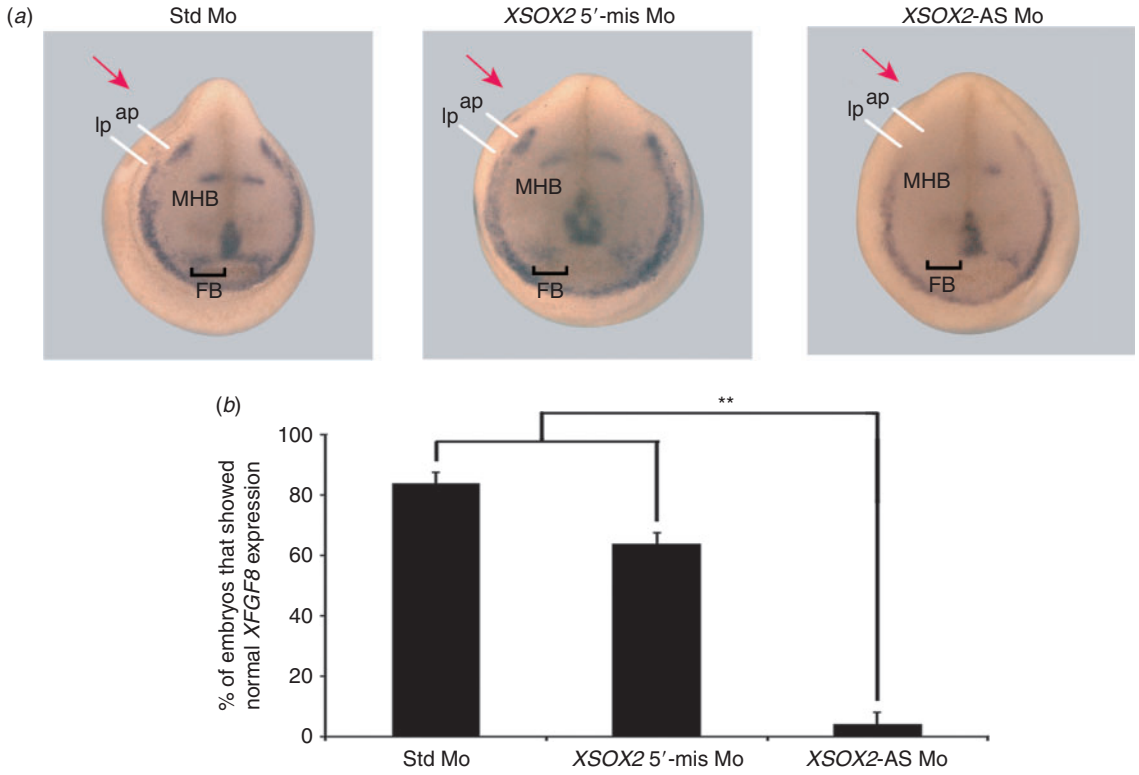

Fig. 5. Effect of *Xenopus* SRY (sex-determining region Y)-box 2 (XSOX2) depletion on *Xenopus* fibroblast growth factor 8 (*XFGF8*) gene expression in early *Xenopus* development. (*a*) Morpholino oligonucleotides (MO) were injected into one blastomere of 2-cell stage *Xenopus* embryos. Expression of *XFGF8* was assessed by whole-mount *in situ* hybridisation. Decreased *XFGF8* expression was noted in the midbrain–hindbrain junction (MHB), auditory placode (ap), lens placode (lp) and forebrain (FB) in *XSOX2* antisense MO (*XSOX2*-AS)-injected sides. The red arrow indicates the side in which MO were injected. (*b*) A decreased in the number of embryos with normal *XFGF8* mRNA expression is evident in *XSOX2*-AS injected *Xenopus* embryos. StdMO, random sequence standard morpholino; *XSOX2* 5′-misMO, 5′-mismatched *XSOX2* MO. Data are the mean ± s.e.m. $^{**}P < 0.01$.

of endogenous *XFGF8* gene (see Fig. S2). These results indicate that the presence of additional regulatory elements or enhancers may be required for proper *XFGF8* expression in the developing *Xenopus* embryo. Furthermore, it may be reasonable to postulate that XSOX2 regulates *XFGF8* gene expression in the presence of other molecules such as homeodomain, zinc finger and basic–loop–helix, as well as leucine zipper proteins (Ambrosetti *et al.* 1997; Kamachi *et al.* 1999; Wissmuller *et al.* 2006). Collectively, the identification of other molecules that regulate *XFGF8* gene expression together with XSOX2 and additional regulatory elements will further clarify the mechanisms responsible for the regulation of *XFGF8* gene expression during early *Xenopus* development.

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