

# Antagonistic role of XESR1 and XESR5 in mesoderm formation in *Xenopus laevis*

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**ABSTRACT** The notch signaling pathway is widely conserved from vertebrates to invertebrates and mediates the specification of numerous cell fates during developmental processes. In the *Xenopus* gastrula embryo, Xdelta1, one of the Notch ligands, is expressed in the prospective mesoderm prior to Xbra expression. Here, we examined the role of Notch signaling in mesoderm formation. Embryos injected with Xdelta1 morpholino oligo DNA showed a severe gastrulation defect and suppression of Xbra expression, which were completely rescued by co-injection with the active form of Notch. In order to fully understand the role of Notch signaling, we examined the expression of the Notch target genes XESR1 and XESR5. RT-PCR and whole-mount *in situ* hybridization analyses showed that XESR5 was highly expressed in the marginal zone of the early gastrula embryo, whereas expression of XESR1 was not detected. Animal cap assays indicated that expression of XESR5 was not induced by Notch signaling but by nodal signaling. To clarify the role of XESR5 in the gastrula embryo, a dominant negative form of XESR5 was injected into the prospective mesoderm. The truncated form of XESR5 induced the ectopic expression of XESR1, which caused a decrease in Xbra expression and defective gastrulation. In contrast, the truncated form of XESR1 caused an upregulation of XESR5 resulting in an increase in Xbra expression. The antagonistic effect of XESR1 and XESR5 suggests a dual regulation in which XESR5 produces a competent area for mesoderm formation by suppressing the gene expression of XESR1, while XESR1 sharpens the boundary of Xbra expression.

KEY WORDS: XESR1, XESR5, *Xenopus*, mesoderm formation

## Introduction

Notch signaling plays a fundamental role during developmental processes in multicellular animals (Artavanis-Tsakonas *et al.*, 1999; Mumm and Kopan, 2000). It mediates communication between adjacent cells and determines the cell fate at the branch point of neuron or glial cell (Hojo *et al.*, 2000), T cell or B cell (Han *et al.*, 2002), and endocrine or exocrine cell (Apelqvist *et al.*, 1999). The Notch receptor is a single transmembrane spanning protein that receives signals from cell-bound ligands of the Delta or Serrate and thus functions by direct cell-cell contact. The Notch receptor undergoes a complex series of proteolytic processing events that lead to the release of the Notch intracellular domain (NICD) (Ebinu and Yankner, 2002). Presenilins are important for the final proteolytic cleavage liberating the NICD (Berezovska *et al.*, 2000; Karlstrom *et al.*, 2002). The cleaved NICD fragment translocates to the nucleus, where it interacts with the DNA-

binding CSL protein (CBF1, Suppressor of Hairless, Lag1 family) to regulate the transcription of target genes (Furukawa *et al.*, 1992; Jarriault *et al.*, 1995). The key downstream genes are the HES family genes encoding basic helix-loop-helix transcription regulators (Iso *et al.*, 2001; Jarriault *et al.*, 1995; Maier and Gessler, 2000; Nakagawa *et al.*, 2000).

In *Xenopus* embryo, the molecular mechanism of Notch signaling is well conserved, and a detailed investigation has been performed in neurogenesis (Chitnis *et al.*, 1995; Wettstein *et al.*, 1997; Koyano-Nakagawa *et al.*, 1999; Kiyota *et al.*, 2001). Prior to the neurula stage, the first zygotic expression of Xdelta1 occurs in the marginal zone of gastrula embryo (Wittenberger *et al.*,

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Abbreviations used in this paper: AP, alkaline phosphatase; DIG, digoxigenin; DN, dominant negative form; MBS, modified Barth saline; MO, morpholino antisense oligonucleotides; NICD, notch intracellular domain.

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1999). An *in vitro* study using animal cap assay has shown that Notch signaling in the marginal zone of gastrula embryo controls cell competence for mesoderm formation (Abe *et al.*, 2005). However, the molecular mechanism by which Notch signaling controls mesodermal competence remains unknown. We have reported that the depletion of XSu(H)2, which functions as a transcription factor under Notch signaling, causes the gastrulation defect (Ito *et al.*, 2007a), suggesting that Notch signaling is required for normal gastrulation. However, the gastrulation defect caused by the depletion of XSu(H)2 could not be rescued by one of the Notch target gene, *Xenopus Enhancer of Split related gene 1* (*XESR1*) (Ito *et al.*, 2007b). Here, we examine the effects of another Notch target gene, *XESR5*, which has been reported in segmentation of the paraxial mesoderm (Jen *et al.*, 1999). RT-PCR and whole-mount *in situ* hybridization showed that *XESR5*, but not *XESR1*, is expressed in the marginal zone of the early gastrula embryo. Animal cap assay indicated that gene expression of *XESR5* in the marginal zone is induced not by Notch signaling but by nodal signaling. Loss- and gain-of-function experiments demonstrated that *XESR1* and *XESR5* antagonistically regulate the gene expression of each other. On the basis of these results, we propose a dual regulation model in which *XESR5* produces a competent area for mesoderm formation by suppressing the gene expression of *XESR1*, while *XESR1* refines the boundary of *Xbra* expression by suppressing the overexpression of *Xbra*.

## Results

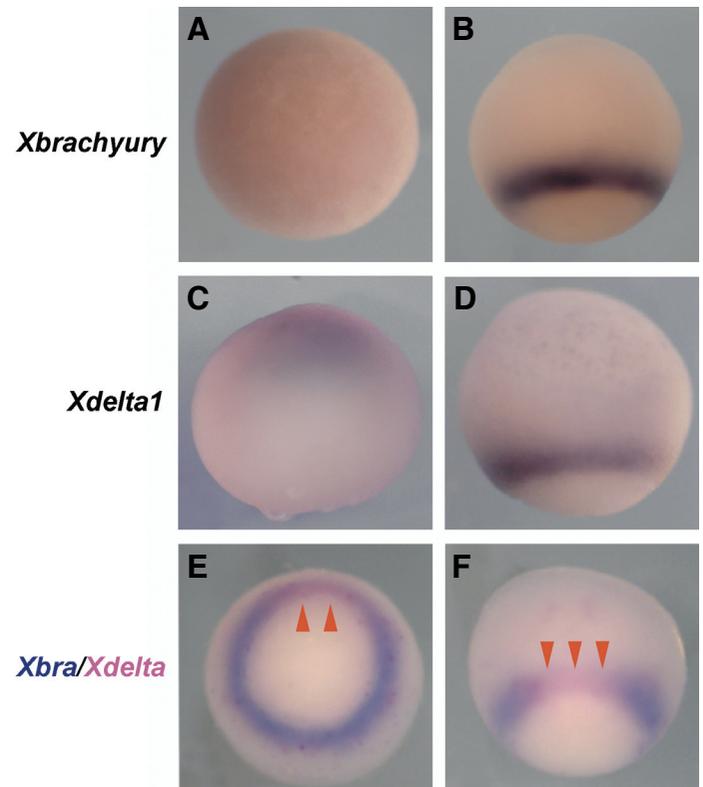
### Effect of Notch signaling on mesoderm formation

It is well established that *Xdelta1* is expressed in the marginal zone of the early gastrula embryo (Wittenberger *et al.*, 1999). To compare the spatiotemporal expression between *Xbra* and *Xdelta1*, whole-mount *in situ* hybridization was performed on the early gastrula. Gene expression of *Xbra* was not detected in the early gastrula embryo, while broad expression of *Xdelta1* was observed in the animal hemisphere of st.9 embryo (Fig. 1 A,C). Thereafter, *Xbra* initiated zygotic expression, and the ring-shaped expression was observed at the late gastrula stage (Fig. 1 B,D). Comparison of the expression areas between *Xbra* and *Xdelta1* revealed that both *Xbra* and *Xdelta1* appear to be expressed in the same area. Double *in situ* hybridization was performed in the st.11.5 embryo to further compare the expression region between *Xdelta1* and *Xbra*. As shown in Fig. 1E and F, gene expression of *Xdelta1* and *Xbra* was detected in the same region of the st.11.5 embryo (Fig. 1 E,F). The co-expression of *Xdelta1* and *Xbra* suggests that *Xdelta1* plays an important role in mesoderm formation. To clarify the role of *Xdelta1* in mesoderm formation, morpholino oligo DNA of *Xdelta1* (*Xdelta1*-MO) was directly injected into the lateral side of one blastomere at the 2-cell stage, and morphogenetic movement was observed at the gastrula stage. In contrast to normal gastrulation in control embryos, the *Xdelta1*-MO-injected embryo showed the gastrulation defect in the injection side (Fig. 2 A,B). The *Xdelta1*-MO-induced gastrulation defect was completely rescued by co-injection with the active form of Notch (NICD) (Fig. 2C). Whole-mount *in situ* hybridization showed that *Xbra* expression was inhibited by *Xdelta1*-MO, which was rescued by co-injection of NICD (Fig.

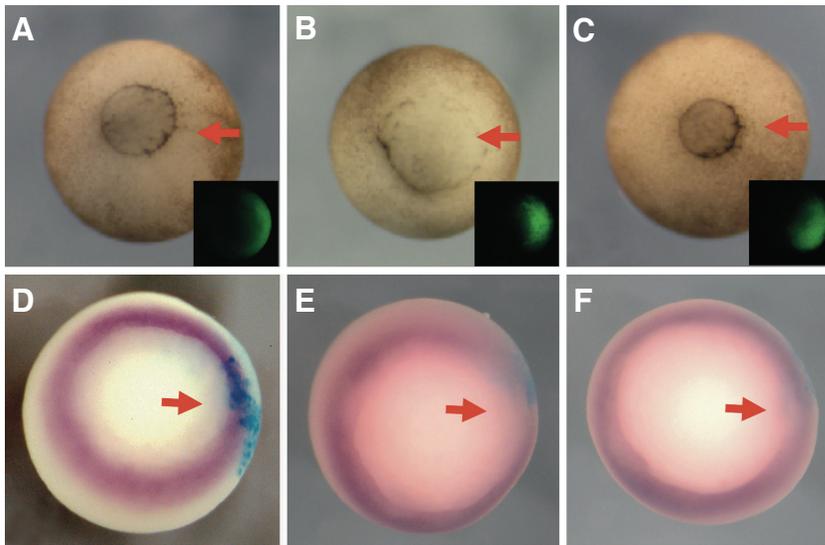
2 D-F). These results indicate that Notch signaling is necessary for mesoderm formation.

### *XESR5* initiates zygotic expression prior to *XESR1* expression

*XESR1* functions as a typical Notch target gene during neurogenesis. To understand the role of Notch target gene in mesoderm formation, gene expression of *XESR1* and *XESR5* was compared during gastrulation. RT-PCR analysis indicated that gene expression of *XESR1* was not detected in the st.10 embryo, while the initial gene expression of *XESR5* was recognized from st.10 (Fig. 3A). In order to clarify the initial stage of the gene expression during mesoderm formation, spatiotemporal expression of both genes was examined by whole-mount *in situ* hybridization. As shown in Fig. 3B, gene expression of *XESR5* was observed in the marginal zone of st.10.5 embryo which showed no expression of *XESR1*. *XESR5* expression is known to be activated by Notch signaling in segmentation of the paraxial mesoderm (Jen *et al.*, 1999). To further determine whether *XESR5* is the target of Notch signaling in the mesoderm formation, animal caps were isolated from the embryo injected with NICD and *Xnr2*, and gene expression was examined by RT-PCR analysis. Surprisingly, results show that gene expression of



**Fig. 1. Comparison of gene expression profiles of *Xbra* and *Xdelta1*.** (A-D) Gene expression of *Xbra* (A,B) and *Xdelta1* (C,D) at st.9 (A,C) and st.11.5 (B,D). All embryos are shown in lateral view with the dorsal side right. *Xdelta1* shows the zygotic expression prior to *Xbra* expression. (E,F) Double *in situ* hybridization of *Xbra* (blue) and *Xdelta1* (red) in the st.11.5 embryo. (E) Vegetal view with the dorsal side up. (F) Dorsal view with the animal side up. Gene expression of *Xbra* and *Xdelta1* overlapped with each other in the same marginal zone. *Xdelta1* expression was recognized in the *Xbra*-free dorsal area (arrowheads).



**Fig. 2. Effect of Xdelta1-MO on gastrulation and Xbra expression.** Xdelta1-MO and tracer mRNAs were injected into the lateral side of one blastomere at the 2-cell stage. Gastrulation (A-C) and Xbra expression (D-F) were examined in the injected embryo at st.12 and st.10.5, respectively. All embryos are shown in vegetal view with the ventral side up, and arrows indicate the injection side. GFP (A-C) and  $\beta$ -gal (D-F) were used as tracers of the injection side. In contrast to the normal gastrulation of the GFP-injected control embryo (A), Xdelta1-MO caused the gastrulation defect in the injection side (B), which was completely rescued by co-injection of NICD (C). Xdelta1-MO inhibited Xbra expression (E), which was rescued by co-injection of NICD (F). Injection of  $\beta$ -gal into control embryos showed no effect on Xbra expression (D).

XESR5 was induced not by NICD, but by Xnr2 (Fig.4, lanes 2–5). In contrast, XESR1 expression was induced not by Xnr2 but by NICD (Fig. 4, lanes 2–5). In the animal cap co-injected with Xnr2 and NICD, XESR1 and XESR5 showed moderate levels of expression (Fig. 4, lane 5). These results suggest that the regulatory mechanism of gene expression is different between XESR1 and XESR5 in mesoderm formation.

**Antagonistic expression of XESR1 and XESR5**

As shown above, Xnr2-induced XESR5 expression was substantially suppressed in the NICD-injected cap (Fig. 4, lane 5). In contrast, NICD-activated XESR1 expression was suppressed in the Xnr2-expressing cap. These results suggest a possibility that XESR1 and XESR5 antagonistically regulate the gene expression of each other. To test this possibility, we examined the relationship between XESR1 gene expression and XESR5 gene expression by using Xnr2-injected animal cap. Gene expression of XESR5 was induced in the Xnr2-expressing animal cap (Fig. 5, lane 2). However, the Xnr2-induced XESR5 was downregulated by co-injection of XESR1 (Fig. 5, lane 3). In contrast, co-injection of the dominant negative form of XESR1 (DN-XESR1) upregulated the Xnr2-induced XESR5 expression (Fig. 5, lane 4). Gene expression of XESR1 was not detected in the Xnr2-expressing animal cap (Fig. 5, lane 2). Co-injection of XESR5 did not cause the ectopic expression of XESR1 (Fig. 5, lane 5). However, the dominant negative form of XESR5 (DN-XESR5) induced a high expression of the XESR1 gene (Fig. 5, lane 6). These results suggest that XESR1 and XESR5 antagonistically regulate the

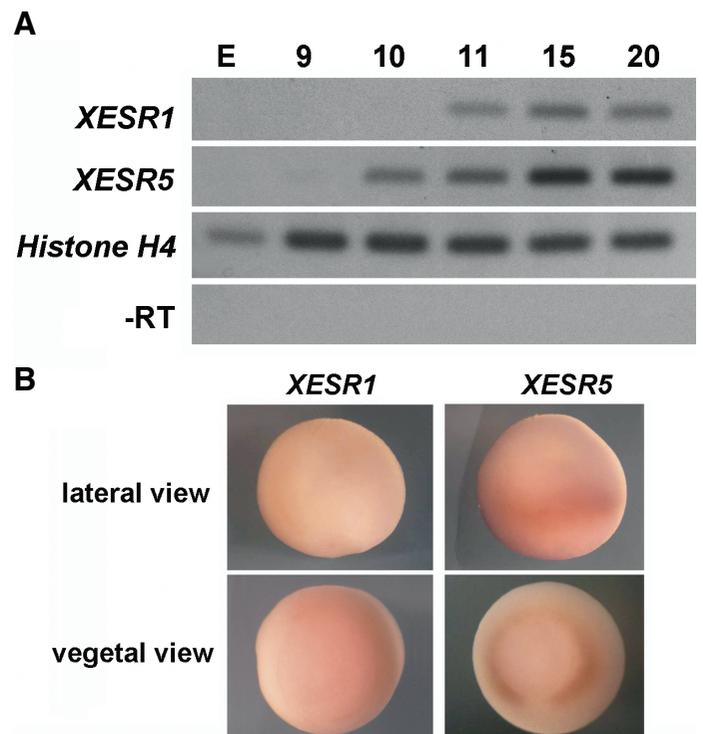
gene expression of each other. To confirm this finding, synthesized RNAs for XESR1 or DN-XESR5 were injected into the lateral side of one blastomere at the 2-cell stage, and gene expression of XESR5 or XESR1 was examined at st.10.5 by whole-mount *in situ* hybridization. Microinjection of XESR1 suppressed the zygotic expression of XESR5 in the injection side, whereas microinjection of DN-XESR5 induced the ectopic expression of XESR1 in the injection side (Fig. 6). These results demonstrated that XESR1 and XESR5 show antagonistic gene expression in the marginal zone of the gastrula embryo.

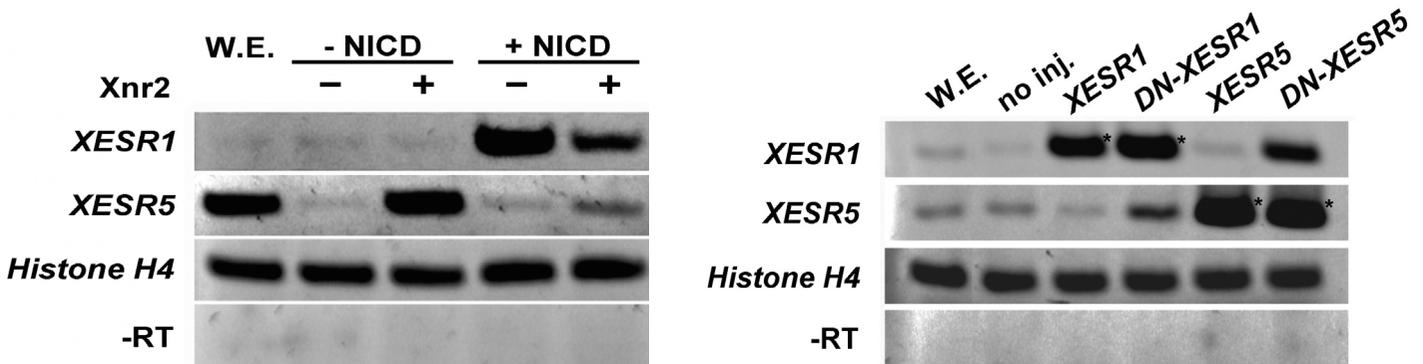
**Role of XESR1 and XESR5 in Xbra expression**

XESR5 showed the ring-shaped expression in the marginal zone of the st.10.5 embryo, but XESR1 did not (Fig. 3B). To further

**Fig. 3. Spatiotemporal expression profile of XESR1 and XESR5.**

(A) Quantitative RT-PCR analysis of XESR1 and XESR5 during early development. Initial gene expression of XESR5 was detected at st.10 and gradually increased up to st.20, whereas initial gene expression of XESR1 was recognized from st.11. E: unfertilized egg. Histone H4 was used as the internal marker. -RT: without reverse transcriptase reaction. Numbers on the photograph indicate the Nieuwkoop and Faber’s developmental stage. (B) Whole-mount *in situ* hybridization of XESR1 (left column) and XESR5 (right column) in the st.10.5 embryo. Upper row shows the lateral view with the dorsal side right, and lower row shows the vegetal view with the ventral side up.





**Fig. 4. (Left) Effect of *Xnr2* and notch intracellular domain (NICD) on the gene expression of *XESR1* and *XESR5*.** Synthesized RNAs of *Xnr2* and/or NICD were injected into the animal side of both blastomeres at the 2-cell stage. Animal caps were isolated from the injected embryo at st.9 and cultured until st.10.5, when gene expression of *XESR1* and *XESR5* was examined by quantitative RT-PCR. Histone H4 was used as the internal marker. -RT: without reverse transcriptase reaction. W.E.: whole embryo.

**Fig. 5. (Right) Antagonistic gene expression of *XESR1* and *XESR5*.** Synthesized RNAs of *Xnr2* and *XESR1*, DN-*XESR1*, *XESR5*, or DN-*XESR5* were injected into the animal side of both blastomeres at the 2-cell stage. Animal caps isolated from the injected embryo at st.9 were cultured until st.10.5, and gene expression of *XESR1* and *XESR5* was examined by quantitative RT-PCR. Large bands marked with asterisk show the total mRNA containing endogenous and exogenous mRNA. Histone H4 was used as the internal marker. -RT: without reverse transcriptase reaction. W.E.: whole embryo. To detect the quantitative difference in *XESR5* expression, the number of PCR cycles was reduced to 24 in this experiment.

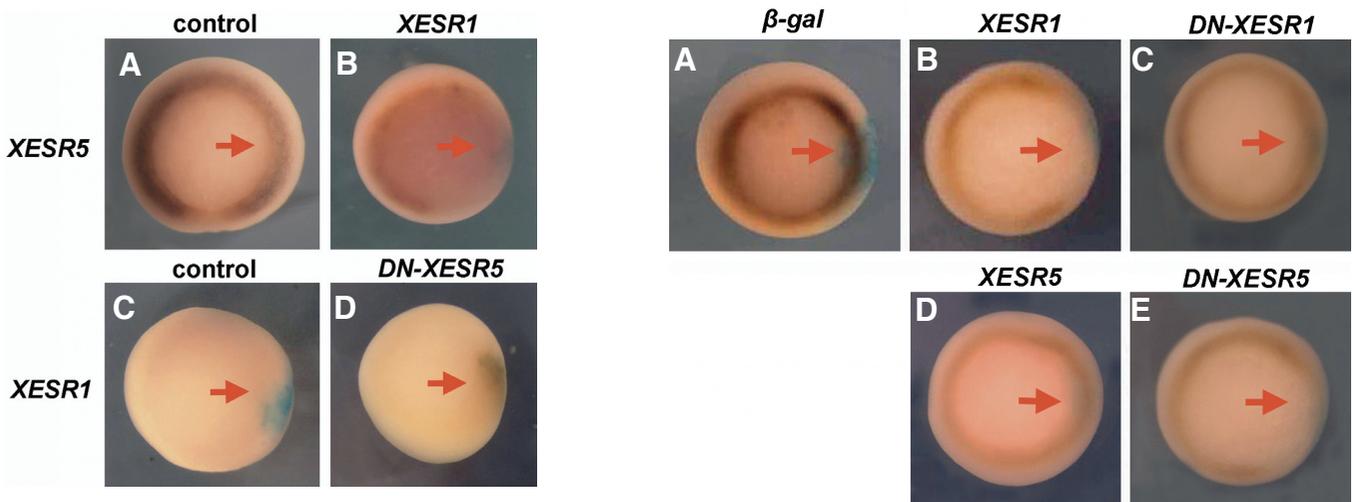
understand the role of *XESR1* and *XESR5* in mesoderm formation, wild type or dominant negative form of *XESR1* or *XESR5* was injected into the lateral side of one blastomere at 2-cell stage, and *Xbra* expression in the injected embryo was examined at st.10.5 by whole-mount *in situ* hybridization. Results show that overexpression of *XESR1* inhibited the *Xbra* expression, whereas DN-*XESR1* induced the upregulation of the *Xbra* expression in the injection side (Fig. 7 A-C). In contrast, overexpression of *XESR5* upregulated *Xbra* expression, while DN-*XESR5* caused

the downregulation of *Xbra* expression in the injection side (Fig. 7 D,E). These results suggest that *XESR1* and *XESR5* may function as antagonists in mesoderm formation.

## Discussion

### Antagonistic expression of *XESR1* and *XESR5*

It has been shown that one of the Notch ligands, *Xdelta1*, is expressed in the marginal zone of early gastrula (Wittenberger et



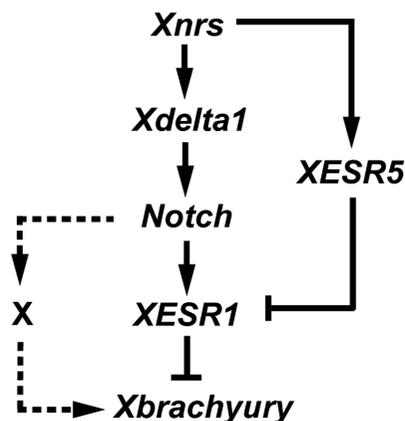
**Fig. 6 (Left). Inhibitory transcriptional regulation of *XESR1* and *XESR5*.** Synthesized RNAs of  $\beta$ -gal (A,C), *XESR1* and  $\beta$ -gal (B) or DN-*XESR5* and  $\beta$ -gal (D) were injected into the lateral side of one blastomere at the 2-cell stage, and gene expression of *XESR5* (A,B) or *XESR1* (C,D) was examined at st.10.5 by whole-mount *in situ* hybridization. All embryos are shown in vegetal view with the ventral side up. Arrows indicate the injection side. *XESR1* inhibited the gene expression of *XESR5* (B, arrow), while DN-*XESR5* induced the ectopic expression of *XESR1* (D, arrow).

**Fig. 7. (Right) Effect of *XESR1* and *XESR5* on the gene expression of *Xbra*.** Synthesized RNAs of *XESR1* (B), DN-*XESR1* (C), *XESR5* (D) or DN-*XESR5* (E) were injected into the lateral side of one blastomere at the 2-cell stage together with  $\beta$ -gal as a lineage tracer, and gene expression of *Xbra* was examined at st.10.5 by whole-mount *in situ* hybridization. Control embryo was injected with  $\beta$ -gal alone (A). All embryos are shown in vegetal view with the ventral side up. Arrows indicate the injection side.

*et al.*, 1999). However, XESR1, one of the Notch target genes, is not expressed in the early gastrula. The first drastic expression of XESR1 has been reported under the Notch signaling in the primary neurogenesis (Wettstein *et al.*, 1997). In the epidermis of the neurula, XESR6e, another HES family gene, has been shown to be involved in the cell fate decision during cilia cell formation (Deblandre *et al.*, 1999). XESR6e is not expressed in the gastrula embryo, but shows the dotted expression pattern in the epidermis of neurula stage embryo. The present study demonstrates that XESR5 is expressed in the marginal zone of early gastrula embryo (Fig. 3B). During myotome formation, XESR5 controls the segmentation of paraxial mesoderm under the Notch signaling (Jen *et al.*, 1999). However, the present study results demonstrate that gene expression of XESR5 is induced not by the Notch signaling but by Xnr2. Loss- and gain-of-function experiments indicate that XESR1 and XESR5 inhibit the gene expression of each other (Fig. 5,6). The antagonistic regulation of the gene expression is consistent with the negative cross-regulation between *hes5* and *hes6* in chick neurogenesis (Fior and Henrique, 2005). The results are also consistent with those obtained in mouse HES family genes where *Hes6* and *Hes1* play an antagonistic role, leading to the periodic segmentation (Bae *et al.*, 2000; Gratton *et al.*, 2003). Taken together, it is likely that Xnr2-induced gene expression of XESR5 occurs prior to the activation of Notch signaling, which results in the suppression of XESR1 expression (Fig. 8).

#### Boundary formation of Xbra expression

In *Xenopus* gastrula embryo, the mesoderm formation occurs in the marginal zone, which is visualized by the zygotic expression of Xbra. Mesoderm-inducing factor such as activin and nodal-related genes induces Xbra expression in a concentration-dependent manner (Gurdon *et al.*, 1994, 1995, 1996). *In vitro* studies using the dissociated blastula cells indicate that the interpretation



**Fig. 8. A dual regulation model showing a hypothetical role of Notch signaling in mesoderm formation.** Zygotic expression of *Xdelta1* and *XESR5* is induced by mesoderm-inducing factors. Since *XESR5* expression initiates prior to activation of Notch, *XESR5* is able to produce a competent area for Xbra expression by inhibiting the gene expression of *XESR1*. *Xdelta1*-activated *XESR1* might play a role to suppress the overexpression of Xbra. Another role of Notch signaling might be to promote Xbra expression by activating a novel target gene X. These transcriptional networks could refine the Xbra-expressing area in the *Xenopus* gastrula embryo.

of concentration of activin by cells is dependent upon the concentration of Smad2 in the nucleus (Bourillot *et al.*, 2002). In addition to the concentration-dependent mesoderm induction, the refinement mechanism of Xbra expression has been reported, which requires protein synthesis (Papin and Smith, 2000). Animal cap assay demonstrates that the boundary formation of activin-induced Xbra expression requires protein synthesis (Kinoshita *et al.*, 2006). However, the type of proteins that control the boundary formation of Xbra expression remains to be determined. In the present study, DN-XESR1 caused the upregulation of XESR5, which increased Xbra expression (Fig. 5,7). These results suggest that the antagonistic regulation of XESR1 and XESR5 is involved in the boundary formation of Xbra expression.

#### Dual role of Notch signaling in Xbra expression

In *Xenopus* development, the essential role of Notch signaling in primary neurogenesis has been well known (Chitnis *et al.*, 1995). Prior to the neurogenesis, the initial zygotic expression of *Xdelta1* occurs in the marginal zone of early gastrula (Wittenberger *et al.*, 1999). Functional analysis of I-Fringe, which is a Notch signal regulator, has indicated that Notch signaling in gastrula embryo is involved in the myogenesis (Wu *et al.*, 1996). In the present study, *Xdelta1*-MO caused the inhibition of Xbra expression and the gastrulation defect, which was completely rescued by co-injecting with NICD. These results suggest that Notch signaling is required for the mesoderm formation. Since XESR1, a canonical Notch target gene, suppresses Xbra expression (Ito *et al.*, 2007a), one possible role of Notch signaling in gastrula embryo might be to refine the boundary of Xbra expression by antagonistic regulation of XESR1 and XESR5. In many aspects of metazoan development, Notch signaling activates or suppresses various non-canonical target genes (Kopan and Ijagen, 2009). In fact, a previous report has demonstrated that Notch signaling in *Xenopus* gastrula embryos regulates the cell competency for the mesoderm inducing factor in Su(H)-independent manner (Abe *et al.*, 2005). Taking together, we can suppose two roles of Notch signaling, one is the inhibiting and another is the activating role in the Xbra expression (Fig. 8). Our previous study using XSu(H)2-MO has indicated that Notch signaling promotes the Xbra expression by activating a novel target gene (Ito *et al.*, 2007a). A potential candidate for the novel target gene is POU family gene (Ito *et al.*, 2007b); however, further studies are necessary for understanding the activating role of Notch signaling in the mesoderm formation.

## Materials and Methods

#### Egg and embryos

*Xenopus* eggs were obtained by injecting human chorionic gonadotropin, gestron (Denka Seiyaku, Japan) into *Xenopus laevis* female and fertilized with the testis isolated from *X. laevis* male by surgical operation. Embryos were dejellied with 2% L-cysteine and cultured in 0.1x MBS (Modified Barth Saline). The developmental stages of embryos were determined according to the normal table of *X. laevis* (Nieuwkoop and Faber, 1967). Animal caps were excised from the blastula embryo at st.8 and cultured in 1x MBS containing 100 mg/L kanamycin until st.10.5, when gene expression in the explants was examined.

#### Constructs and morpholino antisense oligonucleotides

Dominant negative forms of *XESR1* (DN-XESR1) and *XESR5* (DN-

*XESR5*) were constructed according to a previous report (Ito *et al.* 2007b). In these constructs, the carboxyl-terminal WRPW motif (Fisher *et al.*, 1996, Giebel and Campos-Ortega, 1997) was deleted by PCR on a template of *XESR-1/pCS2+* or *XESR-5/pCS2+*, and the PCR product was subcloned at the *Bam*HI/*Xho*I site. According to the manufacturer's instructions (Gene Tools), morpholino antisense oligonucleotides (MO) for *XDelta1* was designed as follows:  
5'-GCGCTGCTGTCCCATGTTGTCTGAT-3'.

#### RNA synthesis and microinjection

All capped mRNAs were synthesized from linearized plasmids using SP6 RNA polymerase (Epicentre Technology). Capped mRNA was made using the mCAP RNA synthesis kit (Gibco BRL) according to the manufacturer's instructions. *NICD* RNA was made from *NICD/pCS2+* (Chitnis *et al.*, 1995), and  $\beta$ -galactosidase RNA was produced from pCMV-SPORT  $\beta$ -gal (Stratagene). Fertilization, culture, and microinjection were performed as described previously (Moon and Christian, 1989, Asashima *et al.*, 1990). One blastomere of a 2-cell-stage embryo was injected with 5 nl solution containing 500 pg mRNA or 30 ng morpholino oligo DNA.

#### RT-PCR analysis

Total RNA was extracted from embryos or animal caps using the Isogen solution (Nippongene). Oligo (dT)-primed first strand cDNA was prepared from 0.5 ng of total RNA using Reverscript1 (Wako, Japan). Each PCR was performed using this cDNA as a template. The RT-PCR conditions were as follows: 95°C for 2 min, 55°C for 2 min and 26 cycles of 72°C 1 min, 95°C 30 sec and 55°C 30 sec. The primer sequences used are as follows:

##### XESR1

Upstream 5'-ACAAGCAGGAACCCAATGTCA-3'  
Downstream 5'-GCCAGAGCTGATTGTTGGAG-3'

##### XESR5

Upstream 5'-TTCATCAGCGAGATCAGTCC-3'  
Downstream 5'-TACAATGGCGGCTGTTTCATG-3'

##### Histone H4

Upstream 5'-CGGGATAACATTCAGGGTATCACT-3'  
Downstream 5'-ATCCATGGCGGTAAGTCTTCT-3'

#### Whole-mount in situ hybridization analysis

Whole-mount *in situ* hybridization was performed according to the improved method of Shain and Zuber (1996). Hybridized probes were visualized according to the Roche Diagnostics DIG protocol, with a minor alteration that 0.45  $\mu$ l NBT (75 mg/ml in dimethyl formamide) and 3.5  $\mu$ l BCIP (50 mg/ml in dimethyl formamide) were added to 1 ml AP buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM MgSO<sub>4</sub>, 0.1% Tween 20, 2.5 mM levamisole). For double *in situ* hybridization, the second probe was labeled with FITC-conjugated UTP instead of DIG-UTP, and the probe was visualized with Magenta (Roche Diagnostics) for AP substrate. The antisense RNA probes of *Xbra*, *Xdelta1*, *XESR1*, and *XESR5* were prepared by linearizing pCS2+ vector with *Eco*RI and transcribed with T7 RNA polymerase.

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