Ventx1.1 competes with a transcriptional activator Xcad2 to regulate negatively its own expression

Shiv Kumar1, Zobia Umair1, Vijay Kumar1, Unjoo Lee2, Sun-Cheol Choi3,* & Jaebong Kim1,*
1Department of Biochemistry, Institute of Cell Differentiation and Aging, College of Medicine, Hallym University, Chuncheon 24252,
2Department of Electrical Engineering, Hallym University, Chuncheon 24252, 3Department of Biomedical Sciences, University of Ulsan College of Medicine, Seoul 05505, Korea

INTRODUCTION

Bone morphogenetic protein 4 (BMP4) is a member of transforming growth factor-β (TGF-β) superfamily of secreted signaling proteins. It controls via Smad1/5/8 the transcription of tissue-specific target genes during early development and adult tissue homeostasis (1). Ventx1.1 (PV.1, Xvent-1b) and Xcad2, homeobox transcription factors, are downstream target genes of BMP4/Smad1 signaling in Xenopus embryos (2, 3). Ventx1.1 acts as a transcriptional repressor to inhibit the expression of neural and organizer-specific genes, including FoxD5b, Zic3, NCAM, Otx2, Gsc, Noggin, and Chordin (2, 4-6). Thus, the ectopic expression of Ventx1.1 causes ventralized and headless embryonic phenotypes. Xcad2 is a transcriptional activator that can induce the expression of BMP4 as well as of its downstream target genes such as Ventx1.1, Ventx1.2, Ventx2.1, and Xpo (3). However, it inhibits the expression of organizer-specific genes Cac, Xlim1, and Otx2 independently of BMP signaling (3). Recently, it has been shown that the BMP4/Smad1 pathway can synergize with the FGF/Xbra pathway to activate Ventx1.1 transcription, resulting in neural inhibition in Xenopus embryos (7, 8). In line with this, 5'-flanking upstream region of Ventx1.1 contains direct cis-acting responsive elements for several transcription factors, including Smad1, Xbra, and OAZ. While the repressive effects of Ventx1.1 on the expression of various neural and organizer genes have been demonstrated, its global binding sites remain to be characterized. Thus, we sought to identify genome-wide occupancy pattern of Ventx1.1 in Xenopus gastrulae using chromatin immunoprecipitation (ChIP)-Seq. As expected, our analysis revealed that neural and organizer-specific genes were included in the list of candidates whose 5'-flanking region might contain binding sites for Ventx1.1. Notably, Ventx1.1 could also bind to its own 5'-flanking upstream sequence. In this study, we have identified direct response element for Ventx1.1 to down-regulate its own transcription in a negative feedback loop. Intriguingly, Ventx1.1 and Xcad2, an activator of Ventx1.1 transcription, competitively co-occupied the common binding site within the 5'-promoter region of Ventx1.1. These results provide an insight into the mechanism by which the expression level of Ventx1.1 is fine-tuned spatially and temporally during dorsoventral patterning of Xenopus embryo.

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RESULTS AND DISCUSSION

Overexpression of Ventx1.1 inhibits the expression of ventral-specific genes

Our ChIP-Seq analysis showed that Ventx1.1 might directly bind to not only its own 5'-flanking sequence but that of Ventx1.2 or Ventx2.1 in Xenopus gastrula-stage embryos (data not shown). Thus, we first examined the effects of the gain-of-Ventx1.1 function on the expression of ventral-specific genes. As shown in Fig. 1A, B, overexpression of Ventx1.1 reduced its own endogenous expression and that of other ventral genes such as Ventx1.2 and Ventx2.1 in animal caps as analyzed by RT-PCR. In contrast, Ventx1.1 had no effect on the expression of BMP4. Given that these ventral genes are BMP4-induced targets (9), these results suggest that Ventx1.1 can down-regulate the expression of ventral-specific genes, including itself, in a BMP-independent manner.

Mapping of Ventx1.1 response element in its own promoter

Negative autoregulation of transcription of a gene can restrict its expression level and/or activity, contributing to proper patterning of embryonic body axis (10, 11). Of potential candidate genes whose 5'-promoter region might contain Ventx1.1-responsive elements, we chose Ventx1.1 itself for this study to gain further mechanistic insight into the regulation of its expression. Analysis of the 5'-flanking genomic sequence (2.5 kb) of Ventx1.1 revealed 13 putative cis-acting response elements for Ventx1.1 (Supplementary Table 1). To identify functional Ventx1.1 response elements (VREs) within the flanking region, we generated luciferase reporter constructs containing serially-deleted Ventx1.1 promoter fragments (Fig. 2A) and then examined the effects of overexpression of Ventx1.1 on their promoter activities. Co-expression of Ventx1.1 significantly reduced the activity of the −2525-bp construct (Fig. 2B), indicating the presence of functional VREs in the full 5'-flanking sequence. Of note, the inhibitory effect of Ventx1.1 on the reporter activity became relatively stronger as gastrulation proceeded (0.7-fold at stage 11.5 as compared with 0.5-fold at stage 10.5). While Ventx1.1-mediated repression of the reporter activity was still observed when the promoter fragment was reduced to −951 bp, this effect was lost upon deletion to −399 bp (Fig. 2C). This suggests that Ventx1.1 response elements are located between −951 bp and −399 bp. The promoter region between −951 bp and −399 bp contains two putative Ventx1.1 binding sites [VRE1, −626 bp to −621 bp (GATTGT) and VRE2, −697 bp to −692 bp (GATTIT)] (Supplementary Table 1). To determine whether these two potential sites could confer Ventx1.1 responsiveness, we substituted 5'-GGGG-3' for the core sequence 5'-ATTT-3' in the putative VRE1 or VRE2 in the −951-bp promoter construct and measured activities of these mutated reporters in the absence or presence of Ventx1.1. Interestingly, mutation of VRE1 (−951 bp-mVRE1), but not of VRE2 (−951 bp-mVRE2),
resulted in a significant decrease (13-fold) in the basal promoter activity of the −951-bp construct even in the absence of Ventx1.1 (Fig. 2D, E), suggesting that the putative VRE1 might also act as a binding site for a transcriptional activator that could positively regulate Ventx1.1 expression. Furthermore, unlike the wild-type −951-bp reporter construct, the −951-bp-mVRE1 reporter was unresponsive to co-injected Ventx1.1 (Fig. 2E), supporting the functionality of VRE1. In contrast, the −951-bp-mVRE2 promoter fragment still exhibited significantly reduced activity upon co-expression of Ventx1.1 (Fig. 2E), indicating that the putative VRE2 might not be relevant to inhibitory autoregulation of Ventx1.1 expression. In addition, we performed ChIP-PCR analysis to test whether Ventx1.1 might indeed bind to the promoter region encompassing VRE1. As shown in Fig. 2F, anti-Flag antibody-mediated ChIP followed by PCR showed that Flag-Ventx1.1 was highly enriched in the promoter fragment containing VRE1. Taken together, these results suggest that Ventx1.1 acts as a repressor of its own transcription by binding to the response element present in its own promoter.

**Xcad2 activates Ventx1.1 transcription by binding to VRE1**

As shown above, the −951-bp promoter with VRE1 mutation displayed significantly reduced basal activity, suggesting that the response element might be co-occupied by a transcriptional activator as well as Ventx1.1 itself as a repressor. It is known that the expression pattern of Xcad2 overlaps with that of Xvent-1 in Xenopus gastrulae and that Xcad2 can positively regulate the expression of ventral-specific genes including Ventx1 and Ventx2 (3, 12). Thus, Xcad2 might be a potential candidate capable of binding to VRE1 to activate Ventx1.1 transcription. As expected, forced expression of Xcad2 increased the transcription of ventral genes such as Ventx1.1, Ventx1.2 and Ventx2.1 and BMP4, in animal caps as analyzed by RT-PCR (Fig. 3A). Conversely, overexpression of Ventx1.1 down-regulated basal and Xcad2-induced levels of expression of ventral genes except BMP4. These results suggest that Xcad2 can act as a transcriptional activator of ventral genes to counteract inhibitory effects of Ventx1.1.

Response elements for caudal-type transcription factors such as Xcad2 have a core consensus sequence, 5'-ATTT-3' (13-15). Note that VRE1 in the promoter region of Ventx1.1 also contains this core sequence (Supplementary Table 1). Consistent with this, overexpression of Xcad2 increased the activity of the −951-bp construct to ~3-fold over the basal level, but had no effect on the activity of its version with VRE1 mutation (Fig. 3B). These results indicate that Xcad2 could up-regulate Ventx1.1 expression by occupying VRE1 within its

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**Fig. 3.** Xcad2 up-regulates Ventx1.1 transcription by occupying VRE1. (A) Animal caps from embryos injected or not with Flag-Ventx1.1 (500 pg) and/or Myc-Xcad2 (1 ng) mRNA as indicated were subjected to RT-PCR analysis at stage 11.5. (B-E) One-cell stage embryos were injected with indicated combinations of −951-bp reporter, −951-bp-mVRE1 reporter, Flag-Ventx1.1 (300 pg), and Myc-Xcad2 (1 ng) and cultured to stage 11 for ChIP-PCR (C, D) or stage 11.5 for luciferase reporter assay (B, E). (D) Quantification of relative intensities of bands in lanes 2, 5, and 6 shown in (C).

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**Fig. 4.** Ventx1.1 and Xcad2 co-occupy Ventx1.1 promoter region in competition with each other. (A-D) Embryos were injected with a combination of Flag-Ventx1.1 (0.5 ng) and increasing doses of Myc-Xcad2 (0.5, 1 ng) or Myc-Xcad2 (1 ng) and increasing doses of Flag-Ventx1.1 (0.5, 0.25, 0.5 ng) as indicated and cultured to stage 11.5 for ChIP-PCR analysis. (B, D) represent quantification of relative intensities of bands in lanes 1, 2, and 3 in (A, C), respectively. (E) A proposed model for Ventx1.1 or Xcad2-mediated control of Ventx1.1 transcription.
promoter region. Thus, we next examined whether Xcad2 might indeed bind to VRE1 to activate Ventx1.1 transcription. As shown in Fig. 3C, overexpressed Xcad2 associated with VRE1 in the promoter region of Ventx1.1 as analyzed by ChiP-PCR. In addition, co-expression of Ventx1.1 caused displacement of Xcad2 from the DNA binding site (Fig. 3C, D), suggesting that VRE1 might be shared by both of the transcription factors. In line with this, overexpression of Xcad2 induced an increase in the activity of the −951-bp promoter fragment, which could be abrogated by co-expression of Ventx1.1 (Fig. 3E). Taken together, these results indicate that Xcad2 binds to VRE1 to up-regulate Ventx1.1 transcription.

**Ventx1.1 and Xcad2 competitively occupy VRE1 to regulate Ventx1.1 transcription**

Co-occupancy of a promoter region by two transcription factors suggests their competition in control of gene expression. Thus, we further investigated whether Ventx1.1 and Xcad2 could interfere with each other in binding to VRE1. As demonstrated by ChiP-PCR experiments, overexpressed Ventx1.1 could bind to its own promoter containing VRE1, while such occupancy by Ventx1.1 was gradually decreased by increasing co-expression of Xcad2 (Fig. 4A, B). In addition, the association between Xcad2 and Ventx1.1 promoter was markedly inhibited by co-expression of Ventx1.1 (Fig. 4C, D). These results suggest that Xcad2 and Ventx1.1 can competitively occupy the cis-acting response element within the promoter region of Ventx1.1 to regulate its transcription in an opposite way.

Ventx1.1 functions as a transcriptional repressor to exert anti-organizer and anti-neural activities, mimicking ventralizing effects of BMP4/Smad1 signaling (2, 4). Ventx1.1 is a direct target of the BMP4/Smad1 pathway. It displays an expression pattern reminiscent of that of BMP4 along the marginal and animal regions of early gastrulae. Expectedly, the cis-acting response elements for Smad1 and a cofactor OAZ as well as positive regulatory factors Xvent-2 and GATA2 have been identified in the promoter region of Ventx1.1 (8, 16). In this study, we also identified Xcad2 as an activator of Ventx1.1 transcription that directly occupied a response element in its promoter sequence (Fig. 4E), which was distinct from that for Smad1, OAZ, or Xvent-2. Mutation of this response element significantly abrogated basal and Xcad2-induced activation of the −951-bp Ventx1.1 promoter, which still contained intact response elements for Smad1, OAZ, and Xvent-2. As such, the contribution of the Xcad2-responsive element to peak activation of the Ventx1.1 promoter would be highly significant. Our previous study has revealed that Xbra, a target of FGF signaling, also cooperates with Smad1 to up-regulate the activity of the Ventx1.1 promoter in a synergistic manner (7). Expression of Xcad2 and Xvent-2 can be induced by not only BMP signal, but also by combined activity of Wnt and FGF signaling pathways (3, 12, 17, 18). Thus, it appears that transcriptional cofactors Xbra, Xcad2, and Xvent-2 can act as mediators of cross-talk between BMP and other signals such as Wnt and FGF in the control of Ventx1.1 transcription. Given that expression patterns of Ventx1.1 and its upstream regulators Xbra, Xcad2, and Xvent-2 in Xenopus gastrulae are overlapped (3), these transcriptional cofactors appear to play roles in restricting Ventx1.1 expression both spatially and temporally. On the other hand, Xbra, Xcad2, or Xvent-2 was unable to efficiently induce Ventx1.1 expression in the absence of BMP signaling (3, 9, 16), indicating the necessity of their cooperation with basic regulatory factors activated by BMP signal. Taken together, these results suggest that these transcriptional factors primarily contribute to maximal activation rather than basal activity of Ventx1.1 transcription.

It has been shown that Goosecoid acts as a repressor of Ventx1.1 transcription to oppose its ventralizing activity (19). Consistently, a response element for Goosecoid is present in the Ventx1.1 promoter, and overexpression of Goosecoid suppresses its activity (8). Since Goosecoid is expressed on the dorsal side of Xenopus early gastrulae, the absence of Ventx1.1 transcription in that region might be primarily due to Goosecoid-mediated repression of its promoter activity. Notably, we observed that Ventx1.1 down-regulates its own transcription by binding to a response element within its own promoter (Fig. 4E). An intriguing aspect of the Ventx1.1 response element in its own promoter is that this sequence is competitively co-occupied by Ventx1.1 and a transcriptional activator Xcad2. This feature suggests that negative auto-regulation of Ventx1.1 transcription occurs in tissues where Ventx1.1 is endogenously expressed such as the ventrolateral marginal region of Xenopus gastrulae. In addition, since Xcad2 is required for the peak activity of Ventx1.1 promoter, it is reasonable to speculate that the inhibitory auto-regulation of Ventx1.1 transcription might be a key regulatory mechanism to maintain Ventx1.1 expression at a moderate level. Overexpression of Ventx1.1 abolished the expression of endogenous Ventx1.1 as well as of its upstream regulator Xvent-2 (Fig. 1), possibly resulting in significant decrease of Ventx1.1 expression below moderate level. As shown in our ChiP-PCR assays (Fig. 4), Ventx1.1 appeared to displace Xcad2 more easily from the co-occupied response element than Xcad2, suggesting a stronger association of Ventx1.1 with the promoter. This strong binding affinity of Ventx1.1 might ensure immediate control of its own expression in the presence of transcriptional activators such as Xcad2. In this respect, fine-tuning of relative cellular levels of Ventx1.1 and Xcad2 might play critical roles in the establishment of optimal level of Ventx1.1 expression, contributing to proper dorsoventral patterning of early embryo. Of note, it has also been shown that Goosecoid can negatively control its own promoter activity for balanced organizer activity (11). Thus, Ventx1.1 and Goosecoid appear to repress each other, leading to their separate expression on the ventral and dorsal sides of embryo, and negatively regulate their own transcription to achieve appropriate levels of their respective expression.
MATERIALS AND METHODS

Embryo manipulation

*Xenopus laevis* were obtained from the Korean *Xenopus* Resource Center for Research. Embryos were obtained by *in vitro* fertilization after induction of female frogs with 500 units of human chorionic gonadotropin (Sigma) as previously described (20). mRNAs and/or DNA constructs were injected into the animal pole region of one-cell stage embryos. For animal cap assays, animal cap explants were dissected from injected or uninjected embryos at stage 8 and incubated in L-15 medium to the desired stages for subsequent experiments.

Ventx1.1 (Xvent-1b, PV.1) genomic DNA and promoter constructs

Genomic library screening for the isolation of Ventx1.1a genomic DNA was previously described (8). A 3.8-kb DNA fragment from the library screening, which contained 2.5 kb of 5'-flanking region of Ventx1.1, was subcloned into pGL-2 basic plasmid (Promega) to produce a −2525-bp promoter construct. Serially-deleted Ventx1.1 promoter constructs were generated by subcloning its promoter fragments, which were obtained from the −2525-bp construct by PCR amplification (Supplementary Table 2), into a pGL-2 basic plasmid.

Site-directed mutagenesis

Mutagenesis was performed using Muta-Direct™ site-directed mutagenesis kit (iNiRON) in accordance with manufacturer’s instructions. Primers used for mutagenesis of VRE1 and VRE2 were as follows: VRE1, (forward) 5'-CAAAG AAGAGGAGG GGGGGGTCGCTGGGGCAA-3' and (reverse) 5'-TTGCCCCAG CGACCCCCCTCCTCTTCAGTG-3'; VRE2, (forward) 5'-CATCC TGCTGGCGGGGGTTCATTCTAG CTG-3' and (reverse) 5'-CAGCTAGAATGACCCCCGCCAGCAGGATG-3'.

RNA synthesis

Capped synthetic mRNAs were *in vitro* generated using a MEGAscript kit (Ambion) according to manufacturer’s instructions. Expression constructs Flag-Ventx1.1 and Myc-Xcad2 were linearized with SacII and Asp718, respectively, and their respective mRNAs were synthesized using SP6 RNA polymerase.

Chromatin Immunoprecipitation (ChIP) assay

For ChIP assay, embryos were injected at one-cell stage with Flag-Ventx1.1 (0.5 ng/embryo) and Myc-Xcad2 (1 ng/embryo) mRNA either alone or together, collected at stage 11 (100 embryos/sample) and processed according to published protocol (21). Anti-Flag (Sigma, F-1804) monoclonal antibody and anti-Myc (Santa Cruz, SC-805) polyclonal antibody were used to immunoprecipitate chromatin fragments. Normal rabbit IgG (Santa Cruz, SC-2027) and mouse IgG (Santa Cruz, SC-2025) were used as negative controls. Primers used for PCRs are listed in Supplementary Table 2 and 3.

RNA isolation and RT-PCR

Total RNA was isolated from whole embryos or animal caps using RNA-Bee reagent (TEL-TEST) and treated with DNase I to remove genomic DNA contamination. cDNAs were synthesized using SuperScript IV (Invitrogen) with 2 μg total RNA per reaction. PCRs were performed as follows: 30 seconds at 94°C, 30 seconds at 57°C and 30 seconds at 72°C; 20-26 cycles of amplification (Supplementary Table 3).

Western blotting

Whole embryos were homogenized in lysis buffer containing phosphatase and protease inhibitors. Proteins were resolved on 12% SDS-PAGE. The following antibodies were used: anti-Flag monoclonal (Sigma, F-1804) and anti-Myc polyclonal (Santa Cruz, SC-805) primary antibodies, and anti-rabbit IgG (Santa Cruz, SC-789) and anti-mouse IgG (Stressgen, SAB-100) secondary antibodies. Protein bands were visualized using an ECL detection kit (GE healthcare).

Luciferase reporter assay

Luciferase reporter assays were carried out using luciferase assay system (Promega) according to the manufacturer’s instructions. Five different groups of embryos (3 embryos/group) were harvested and homogenized in lysis buffer (10 μl/embryo). Embryo lysates (10 μl) were assayed with luciferase substrate (40 μl), and relative luciferase activity was determined by luminometer (EG & G, Berthold). Independent experiments were repeated at least three times.

Statistical analysis

All data were analyzed using GraphPad Prism4. Statistical analysis was performed with one-way analysis of variance (ANOVA). A P value of less than 0.05 was considered statistically significant (**P < 0.01; ***P < 0.001; n.s., not significant).

Nucleotide sequence accession number

Ventx1.1 cDNA sequence was deposited at GenBank (accession number: AF133122).

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

REFERENCES

1. Massague J (2008) TGFbeta in Cancer. Cell 134, 215-230
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2. Ault KT, Dirksen ML and Jamrich M (1996) A novel homeobox gene PV.1 mediates induction of ventral mesoderm in Xenopus embryos. Proc Natl Acad Sci U S A 93, 6415-6420

3. Pillemer G, Yelin R, Epstein M et al (1998) The Xcad-2 gene can provide a ventral signal independent of BMP-4. Mech Dev 74, 133-143

4. Ault KT, Xu RH, Kung HF and Jamrich M (1997) The homeobox gene PV.1 mediates specification of the prospective neural ectoderm in Xenopus embryos. Dev Biol 192, 162-171

5. Hwang YS, Lee HS, Roh DH et al (2003) Active repression of organizer genes by C-terminal domain of PV.1. Biochem Biophys Res Commun 308, 79-86

6. Hwang YS, Seo JJ, Cha SW et al (2002) Antimorphic PV.1 causes secondary axis by inducing ectopic organizer. Biochem Biophys Res Commun 292, 1081-1086

7. Kumar S, Umair Z, Yoon J et al (2018) Xbra and Smad-1 cooperate to activate the transcription of neural repressor ventx1.1 in Xenopus embryos. Sci Rep 8, 11391

8. Lee HS, Lee SY, Lee H et al (2011) Direct response elements of BMP within the PV.1A promoter are essential for its transcriptional regulation during early Xenopus development. PLoS One 6, e22621

9. Yoon J, Kim JH, Lee SY et al (2014) PV.1 induced by FGF-Xbra functions as a repressor of neurogenesis in Xenopus embryos. BMB Rep 47, 673-678

10. Charney RM, Paraíso KD, Blitz IL and Cho KWY (2017) A gene regulatory program controlling early Xenopus mesendoderm formation: Network conservation and motifs. Semin Cell Dev Biol 66, 12-24

11. Danilov V, Blum M, Schweickert A, Campione M and Steinbeisser H (1998) Negative autoregulation of the organizer-specific homeobox gene goosecoid. J Biol Chem 273, 627-635

12. Keenan ID, Sharrard RM and Isaacs HV (2006) FGF signal transduction and the regulation of Cdx gene expression. Dev Biol 299, 478-488

13. Ryu JH, Nam KB, Oh CT et al (2004) The homeobox gene Caudal regulates constitutive local expression of antimicrobial peptide genes in Drosophila epithelia. Mol Cell Biol 24, 172-185

14. Hwang MS, Kim YS, Choi NH et al (2002) The caudal homeodomain protein activates Drosophila E2F gene expression. Nucleic Acids Res 30, 5029-5035

15. Suh E, Chen L, Taylor J and Traber PG (1994) A homeodomain protein related to caudal regulates intestine-specific gene transcription. Mol Cell Biol 14, 7340-7351

16. Friedle H and Knochel W (2002) Cooperative interaction of Xvent-2 and GATA-2 in the activation of the ventral homeobox gene Xvent-1B. J Biol Chem 277, 23872-23881

17. Onichtchouk D, Gawantka V, Dosch R et al (1996) The Xvent-2 homeobox gene is part of the BMP-4 signalling pathway controlling [correction of controlling] dorsoventral patterning of Xenopus mesoderm. Development 122, 3045-3053

18. Hikasa H, Ezan J, Itoh K, Li X, Klymkowsky MW and Sokol SY (2010) Regulation of TCF3 by Wnt-dependent phosphorylation during vertebrate axis specification. Dev Cell 19, 521-532

19. Sander V, Reversade B and De Robertis EM (2007) The opposing homeobox genes Goosecoid and Vent1/2 self-regulate Xenopus patterning. EMBO J 26, 2955-2965

20. Park DS, Kim K, Jang M and Choi SC (2018) Role of dipeptidyl peptidase-4 as a potentiator of activin/nodal signaling pathway. BMB Rep 51, 636-641

21. Blythe SA, Reid CD, Kessler DS and Klein PS (2009) Chromatin immunoprecipitation in early Xenopus laevis embryos. Dev Dyn 238, 1422-1432