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Activin/Nodal signals mediate the ventral expression of *myf-5* in *Xenopus* gastrula embryos☆

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Abstract

Expression of *myf-5*, a key component of myogenic regulatory genes, expands into the ventral marginal zone during *Xenopus* gastrulation after the dorsal activation takes place. Little is known about how this dynamic expression pattern occurs. Here, we provide evidences to suggest that Activin/Nodal signals participate in the regulation of ventral expression of *Xmyf-5* in gastrula embryos. Two Smad binding elements (SBEs) within the *Xenopus myf-5* promoter can specifically interact with Smad4 protein. Furthermore, we demonstrate that the two SBEs are both indispensable for conferring responsiveness to Activin/Nodal signals and to ventral expression of *myf-5* in *Xenopus* gastrula embryos.

Keywords: Activin/Nodal signals; Smad binding element; *Xenopus myf-5*; Myogenesis

The myogenic regulatory factors (MRFs), a group of basic helix–loop–helix (bHLH) transcription factors including Myf-5, MyoD, myogenin, and MRF4, play critical regulatory functions in the skeletal muscle differentiation [1]. *Myf-5* and *myoD*, both expressed before *myogenin* and *MRF4*, are considered to play key roles in the activation of myogenesis [2]. Genetic and tissue culture studies demonstrate that Myf-5, via sharing redundant functions with MyoD, is important in the activation of skeletal muscle specific genes both in vivo and in vitro [3].

The expression pattern of *myf-5* is thought to be consistent with its role in the early determination of myogenic lineage. In *Xenopus*, *Xmyf-5* transcription initiates in the region above the dorsal lip of stage 10 embryos. As gastrulation goes on, its expression domain quickly shifts to the dorsolateral marginal zone and then further expands to the ventrolateral marginal zone [4,5]. A T-box binding site has been previously identified to mediate the dorsal activation of *Xmyf-5* [6]. However, the mechanism that is responsible for the ventral expression of *Xmyf-5* during gastrulation remains unknown. Moreover, according to the revised fate map of *Xenopus* gastrula embryo [7–10], the cells of ventral marginal zone where *Xmyf-5* is expressed will later develop into caudal somites rather than the ventral cell types. Thus, it would be of great interest to investigate the mechanism of expansion of *Xmyf-5* expression from dorsal to ventral marginal zone.

Activin and Nodal-related factors, members of the TGF-β superfamily, are mesoderm-inducing factors [11]. It has been demonstrated that Nodal signals activate the same or a highly related pathway as Activin does [12]. The transmission of this TGF-β superfamily signal from cell surface to nucleus is mediated by Smad proteins, which can positively or negatively regulate the transcription of target genes with other partners such as FAST (forkhead Activin signal transducer) proteins [13]. *Nodal* deficient mice lack a primitive streak and most mesoderm [14]. In zebrafish, double mutants for *nodal*-related genes *cyclops* and *squint* are devoid of head and trunk mesoderm [15]. In *Xenopus*, five different

☆ *Abbreviations:* TGF-β, transforming growth factor-β; SBE, Smad binding element.

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mesendoderm-inducing Nodal-related factors (Xnr1, 2, 4, 5, and 6) have been identified [16]. The expression of endogenous Nodal-related factors starts from dorsal side and then expands to the ventral marginal zone during later gastrulation [17]. This expression pattern strongly suggests that Nodal signal input may be relevant to the regulation of *Xmyf-5* transcription in the ventral marginal zone of gastrula embryos, from which the caudal somites of later stage embryos arise.

In this report, we evidently demonstrated that Activin/Nodal signals mediate the ventral expression of *myf-5* in gastrula embryos via two Smad binding elements (SBEs) within the *Xmyf-5* promoter.

Materials and methods

Constructs. Series deletion constructs p-4223Luc, p-3798Luc, and p-3384Luc were generated by inserting various lengths of the *Xmyf-5* promoter region of plasmid FL-SK into the pGL3-basic vector with *Sac*I/*Hin*dIII, *Apa*I/*Hin*dIII, and *Bgl*II/*Hin*dIII restriction enzyme digestion [18]. Construct p-3601Luc was generated by PCR amplification: PCR products amplified with primer-1 and primer-2 were digested with *Sac*I and *Bgl*II and inserted into plasmid p-3384Luc. Primers used were: 1, 5′-attagagctcaccccaaatggacag-3′ and 2, 5′-ctatcagatctttttttccactggtc-3′. The restriction enzyme sites introduced were underlined. Construct p-4223/-3798 was constructed by inserting the sequence from pos. −4223 to pos. −3798 upstream into the pGL-3 vector.

Three mutant constructs ($pmSBE_1Luc$, $pmSBE_2Luc$, and $pmSBE_{1+2}Luc$) were achieved by using PCR-based overlap extension strategy described in [19]. The primers are shown below for mutagenesis. primer $mSBE_1F$: 5′-aatgtattgctcgtg**gggccc**atccacttctcagtttagat-3′; primer $mSBE_1R$: 5′-atctaaactgagaagtggat**gggccc**cacgagcaatacatt-3′; primer $mSBE_2F$:5′-ctcctgaacgtgtatg**gggccc**ctgggtagcaggaactagc-3′; primer $mSBE_2R$:5′-gctagttcctgctacccag**gggccc**catacacgttcaggag-3′; primer3: 5′-gaaaaggagctcccactccaaac-3′; and primer4: 5′-ctatcagatctttttttccactggtc-3′. Mutated nucleotides are shown in bold. For GFP reporter constructs, the Luc fragment was replaced with *GFP* cDNA.

Xenopus embryo manipulation, RT-PCR, and luciferase assay. Eggs were obtained from *Xenopus* females, fertilized in vitro, and cultured as described previously [20], while embryos were staged according to Nieuwkoop and Faber [21]. For RT-PCR assays, total RNA was prepared using Trizol system and reversely transcribed into cDNA with Superscript II system (Gibco). Primers for PCR were as in [22,23]. For episomal reporter assays, 2 nl of solution containing 20 pg of the various luciferase test constructs and 20 pg of the reference reporter construct pRL-SV40 (Promega), was injected into the animal pole of a two-cell stage embryo or dorsal–lateral marginal zone of four-cell stage embryos. Luciferase assays were performed at stage 12.5, using the Dual-Luciferase Reporter Assay System (Promega). For Activin/Xnr-1 induction assays, reporter constructs with or without various amounts of *Xnr-1* mRNA were injected into the animal pole of two-cell stage embryos and animal caps were dissected at stage 8.5. Explants were then divided into two groups (3 × 5 caps/group), either treated with Activin for 1 h or incubated in 1× MBS and then harvested at stage 12.5 for luciferase assays. Each result was repeated at least three times independently.

Transgenesis and in situ hybridization. Transgenic *Xenopus* embryos were generated using restriction enzyme-mediated integration (REMI) approach as described by Kroll and Amaya [24]. Plasmids used for transgenesis were linearized with *Not*I digestion. In situ hybridizations were performed as described [25].

Electrophoresis mobility shift assay. The Smad binding element (SBE) probes for EMSA were end-labeled by standard method. The single strand sequences of the probes are: 5′-tgtattgctcgttgtgctatcca-3′ (SBE_1), 5′-tgtatgtgtctctgggtagcagga-3′ (SBE_2). 5′-aatgtattgctcgtg**gggccc**atccacttctcagtttagat-3′ ($mSBE_1$), and 5′-ctcctgaacgtgtatg**gggccc**ctgggtagcaggaactagc-3′ ($mSBE_2$). Mutated sites are shown in bold. Nuclear extracts were prepared from stage 11 gastrula embryos and EMSA was performed as described [26]. Ten microliters of nuclear extracts was used in each reaction. Smad4 protein was in vitro synthesized using Promega TNT SP6 reticulocyte lysate system and 2 μL equivalents of reticulocyte lysate was used in each reaction.

Results

Xmyf-5 induction by Activin/Nodal signals

We have previously reported that a 839 bp DNA fragment between pos. −4223 and pos. −3384 of the *Xmyf-5* promoter is essential for the full ventral expansion of the *myf-5* expression domain [18]. In *Xenopus* gastrula embryos, after its onset on the dorsal side, the expression of nodal-related genes (*Xnrs*) also expands to ventral marginal zone [17], overlapping the *Xmyf-5* positive domain. This expression pattern makes Xnrs potential candidates in mediating the ventral expansion of *Xmyf-5*. To address this possibility, we first injected *Xnr-1* mRNA into the animal pole of two-cell stage embryos. The animal caps were dissected at stage 8.5 and harvested at stage 11 for RT-PCR analysis. The PCR results demonstrated that Xnr-1 could induce the expression of *Xmyf-5* and *XmyoD* in the animal caps (Fig. 1A), which is in a dose-dependent manner (data not shown). Moreover, this induction was abolished in the animal caps co-injected with *Cer-S* mRNA, the specific antagonist against Xnr-1 [27]. These results resembled effects obtained by Activin-treated animal caps (Fig. 1A).

In vivo, injection of *Xnr-1* mRNA into the ventral marginal zone of four-cell stage embryos induced ectopical *Xmyf-5* expression in the ventral marginal zone of gastrula embryos (Figs. 1B and C). Ectopically expressed Cer-S in the dorsal–lateral marginal zone, as expected, could block the expression of *Xmyf-5* in gastrula embryos (Figs. 1D and E). Taken together, these results suggest that Activin/Nodal-related signals participate in the regulation of *Xmyf-5* expression.

The ventral expansion of Xmyf-5 expression in Xenopus gastrula embryos

To study the role of Activin/Nodal signals in the regulation of *Xmyf-5* expression, we focused on identifying the sequences of the *Xmyf-5* promoter that responds to Activin/Nodal signaling. We generated four serial deletion constructs according to the 839 bp sequence of the *Xmyf-5* promoter that was responsible for the ventral expansion of *Xmyf-5* expression in gastrula embryos (Fig. 2A). Each reporter construct was

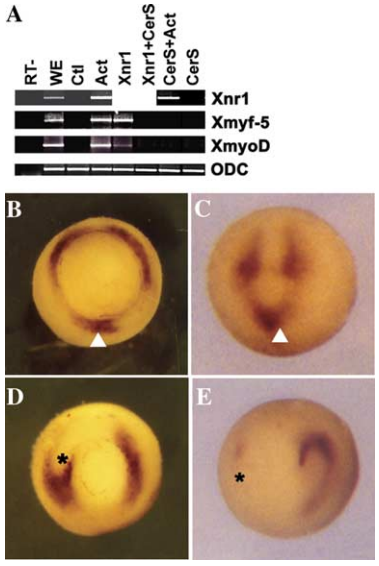

Fig. 1. Induction of *myf-5* by Activin/Nodal signals in *Xenopus* embryos. (A) Both Activin and Xnr-1 could induce *Xmyf-5* and *XmyoD* expression in animal caps. Induction of *Xmyf-5* and *XmyoD* by 100 pg Xnr-1 was inhibited by co-injection with *Cer-S* mRNA. *Xmyf-5* and *XmyoD* expression by Activin treatment was also inhibited when the animal caps were injected with *Cer-S* mRNA, in which *Xnr1* mRNA was induced but its function was inhibited specifically by Cer-S. RT-, non-reverse-transcriptase control; WE, whole embryo (stage 12.5); Ctl, control animal caps without Activin treatment; Act, Activin-treated (100 ng/ml, 1 h) animal caps; ODC, ornithine decarboxylase, as loading control. (B,C) Induction of *Xmyf-5* (marked by ▲) in embryos injected with 100 pg of *Xnr-1* mRNA at four-cell stage at the dorsal–lateral marginal zone. B is a stage 11$^-$ embryo and C is a stage 12.5 embryo showing the expression of *Xmyf-5* in embryos receiving *Xnr1* mRNA injection. (D,E) *Cer-S* mRNA injection (100 pg in D, 400 pg in E) in the dorsal–lateral marginal zone inhibits *Xmyf-5* expression ingastrula embryos (marked by *). Embryos were viewed from the vegetal side, with dorsal side up.

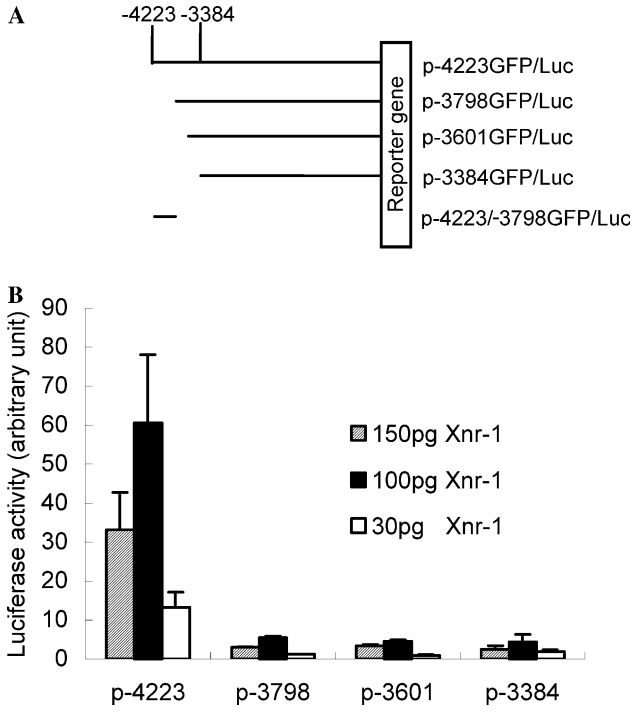

Fig. 2. The sequence between pos. −4223 and pos. −3798 is required for the Activin/Nodal response in reporter gene activity driven by *Xmyf-5* promoter. (A) Diagram of *Xmyf-5* promoter deletion constructs used in this study. (B) Relative luciferase activities. Three doses (30, 100, and 150 pg) of *Xnr-1* mRNA was co-injected, respectively, with reporter gene constructs in the same round of experiment. Fold induction of reporter gene activity was measured by the relative light unit of Xnr-1 induction caps over that of non-induction caps. Xnr-1 response of construct p-4223Luc was above six times higher than that of the other three shorter constructs. Three rounds of independent experiments were performed with similar results.

co-injected with *Xnr-1* mRNA into the animal pole of two-cell stage embryos. As Xnr-1/Activin both could induce *Xmyf-5* expression in a dose-dependent manner (data not shown), we performed the luciferase reporter gene assay with three different strengths of Xnr-1 induction in each single round of experiment. Animal caps were dissected at the middle blastula stage and then cultivated to stage 12.5 for luciferase activity measurement, when endogenous *Xmyf-5* level peaks. As shown in Fig. 2B, three strengths of Xnr-1 induction all demonstrated that p-4223Luc reporter gene construct rendered the highest responsiveness to Xnr-1 signal, most sharply when 100 pg *Xnr-1* mRNA was applied. Further deletion analysis failed to narrow down even smaller DNA fragment that is capable of conferring full Nodal signal responsiveness. Likewise, similar reporter gene responsiveness was achieved in Activin-treated animal caps (data not shown). We therefore concluded that the fragment between pos. −4223 and pos. −3798 of *Xmyf-5* promoter could fully respond to Activin/Nodal signals.

Of particular interest was whether the fragment responding to Activin/Nodal signals could mediate the ventral expression of *Xmyf-5* in gastrula embryos. To address this question, we introduced GFP reporter constructs driven by series deletions of the *Xmyf-5* promoter region (see Fig. 2A) into the embryos by transgenesis technique. Reporter gene expression was detected by in situ hybridization in transgenic embryo with specific *GFP* probe. Compared to the expression pattern of endogenous *Xmyf-5* in normal gastrula embryos (Fig. 3E), *GFP*-positive signals in transgenic embryos surrounded the blastopore as a ring in p-4223GFP (Fig. 3A; 63%, $n = 50$), which contains the DNA fragment responding to Activin/Nodal signals. However, in the transgenic embryos of p-3384GFP, p-3601GFP, and p-3789GFP constructs that are all devoid of the Activin/Nodal responding fragment, *GFP* expression remained in the dorsal half of the marginal zone (Fig. 3C, 76%, $n = 151$). These results demonstrated that the fragment responding to Activin/Nodal signals was required for the ventral expansion of *Xmyf-5* in *Xenopus* gastrula embryo. Furthermore, consistent with the observations that the ventral marginal zone of

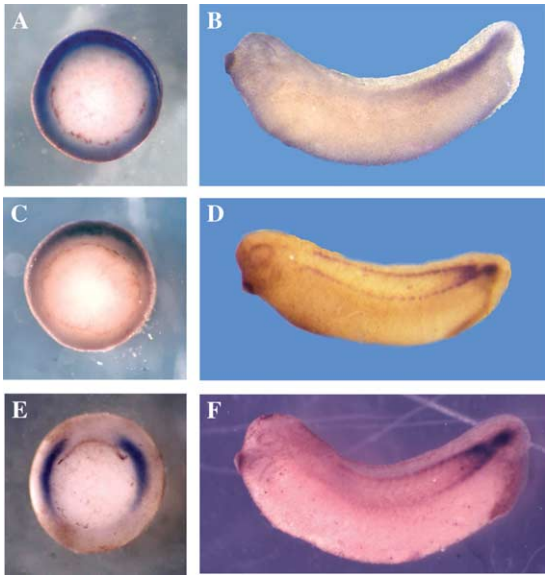

Fig. 3. The ventral expansion of reporter gene expression in gastrula embryos and the reporter gene expression in the posterior trunk of tailbud stage embryos. The reporter gene expression in transgenic embryos was detected by in situ hybridization with *GFP* probe, while endogenous expression of *myf-5* with *Xmyf-5* probe. At stage 11, *GFP* expression in transgenic embryos with p-4223GFP extended to the whole marginal zone (A), while restricted in the dorsal marginal zone in embryos with other three shorter constructs ((C), p-3798GFP as representative). At stage 31, *GFP* expression appeared in the posterior trunk somites in construct containing the Activin/Nodal response fragment ((B), p-4223GFP). While in the constructs lacking this fragment, *GFP* expression in the posterior somites was not detected, even with prolonged staining ((D), p-3798GFP, as a representative). (E) and (F), respectively, represented endogenous *Xmyf-5* expression in stage 11 and stage 31. (A, C, and E) vegetal view, with dorsal up. (B, D, and F) Lateral view, with anterior to left.

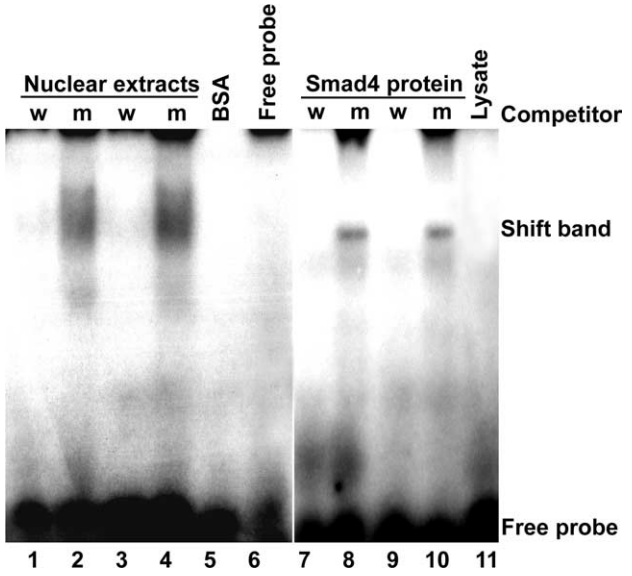

Fig. 4. The two Smad binding elements (SBEs) within the *Xmyf-5* promoter region can specifically interact with nuclear proteins of gastrula embryos and Smad4 protein. The sequence from −3858 to −3849 (SBE_1, lanes 1, 2, 7, and 8) and from −4133 to −4114 (SBE_2, lanes 3, 4, 9, and 10) that encompassed the SBE were radiolabeled and incubated with nuclear extracts of stage 11 embryos or in vitro synthesized Smad4 protein. Formation of the DNA–protein complex was inhibited by competition with 100-fold molar excess of wide type probes (denoted as w), while not affected by competition with DNA with mutated SBEs. Lane 5, BSA (bovine serum albumin) alone, as control; Lane 6, free probe alone; and Lane 11, TNT reticulocyte lysate system without DNA, as a control.

gastrula embryos developed to the caudal tissues rather than ventral cell types [7,10], reporter gene activity of p-4223GFP constructs was detected in the posterior somites at tailbud stage transgenic embryos. In contrast, reporter gene activities of the other constructs were only restricted in the tissues of tailbud and the most posterior presegmental mesoderm, with epaulette shape expression pattern lost (Figs. 3B, D, and F).

Smad binding elements in the Activin response fragment

Activin/Nodal pathway acts primarily through Smad proteins, particularly Smad2, to propagate signals to the target genes [28–31]. Receptor-activated Smads (R-Smads), once phosphorylated, dissociate from the receptor, bind to Smad4 (a Co-Smad), and enter the nucleus to bind to DNA [13]. We found two Smad binding elements (SBEs) located in the fragment that responds to Activin/Nodal signals and mediates the ventral expansion of reporter gene expression in gastrula embryos, one in the pos. −3844 (designated as SBE_1) and the other in the pos. −4126 (SBE_2).

By electrophoresis mobility shift assay (EMSA), we observed that the oligonucleotides containing SBE site as labeled probes were capable of interacting with nuclear extracts of stage 11 embryos. However, no binding complex was observed by pre-incubating with unlabeled wide type probes (denoted as w in Fig. 4, lane 1 for SBE_1, lanes 3 for SBE_2), while competition with unlabeled SBE mutant probes (denoted as m) had no effect on the formation of DNA–protein complex (Fig. 4, lanes 2 and 4), indicating that the two SBEs can specifically interact with gastrula nuclear proteins. Furthermore, both of SBEs could be recognized by the in vitro synthesized Smad4 protein (lanes 7–11). However, the two SBEs failed to be bound by Smad2 protein (data not shown), consistent with the observations that the most common splice form of Smad2 lacks DNA-binding activity [31]. Nevertheless, the EMSA results implicated that Activin/Nodal signals play a role in the regulation of *myf-5* expression in the ventral marginal zone of *Xenopus* gastrula embryos.

The indispensability of two SBEs to ventral expression of myf-5 in Xenopus gastrula embryos

Given the significance of Smad protein in the Activin/Nodal signal pathway and the indication of EMSA results, we performed a series of mutation analyses to

attest the potential role of two SBEs within the identified 425 bp DNA fragment of *Xmyf-5* promoter in mediating the ventral expression of *Xmyf-5* (with constructs diagramed in Fig. 5A). Based on the same condition of animal cap assay performed above, co-injecting $pmSBE_1Luc$ (in which SBE_1 was mutated) or $pmSBE_2Luc$ (in which SBE_2 was mutated) with *Xnr-1* mRNA in the animal pole of two-cell stage embryos resulted in three and two times lower fold induction than that of p-4223Luc, respectively (Fig. 5B). When we co-injected *Xnr-1* mRNA with $pmSBE_{1+2}Luc$ in which two SBEs were both destroyed, its fold induction sharply reduced to nearly 15% of p-4223Luc (Fig. 5B). Consistent with this observation, co-injection of these reporter gene constructs, respectively, with *Xnr-1* mRNA in the dorsal–lateral marginal zone of four-cell stage embryos, where endogenous *Xmyf-5* expresses during gastrulation, demonstrated that $pmSBE_{1+2}Luc$ exhibited the lowest responsiveness to Xnr-1 induction (about four times lower than that of p-4223-Luc, Fig. 5C). Thus, mutational analyses by luciferase assays demonstrated that the SBEs within the *Xmyf-5* promoter are both required for the Activin/Nodal signals.

Next, we carried out transgenesis experiments to further address whether the two SBEs could specifically mediate *Xmyf-5* ventral expression in gastrula embryos. As expected, the *GFP* positive signal was evidently restricted to the dorsal region of marginal zone along the blastopore in transgenic embryo with the $pmSBE_{1+2}GFP$ construct (Fig. 5F, 88%, $n = 50$), compared to a full ring pattern of *GFP* signal in transgenic embryo with p-4223GFP (see Fig. 3A). At tailbud stage, *GFP* expression, likewise, remained at the tailbud region in transgenic embryos with $pmSBE_{1+2}GFP$ (as in Fig. 5G). However, when we introduced either of single SBE site-mutated reporter gene constructs in the *Xenopus* embryo, *GFP* signals were wider in transgenic embryos than in those with $pmSBE_{1+2}GFP$, extending to the ventral

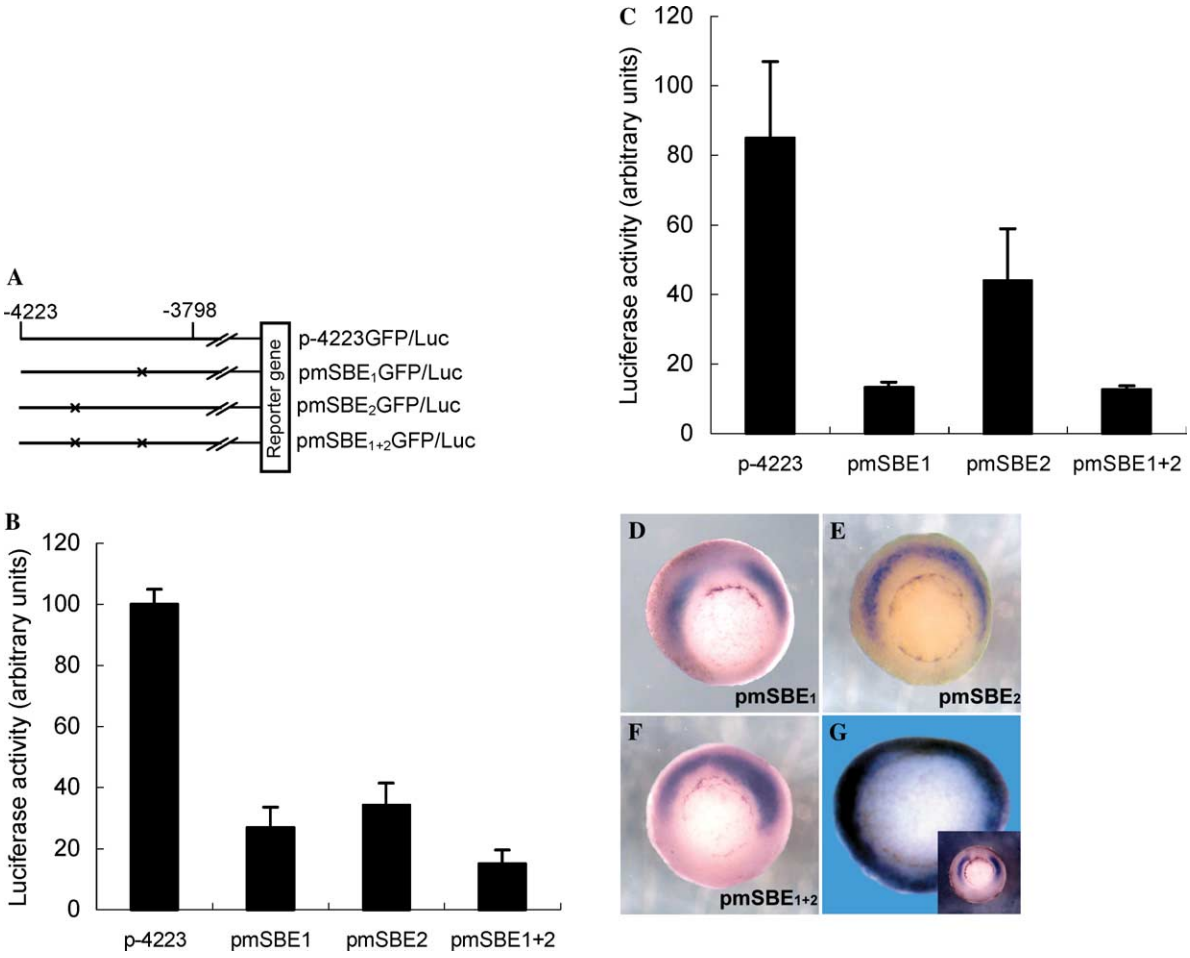

Fig. 5. The two SBEs are indispensable to conferring Activin/Nodal responsiveness and ventral expression of *Xmyf-5* during gasrulation. (A) Diagram of SBE site-mutated constructs of *Xmyf-5* promoter used. Results of Luciferase assay of episomal reporter gene injection in animal pole with 100 pg *Xnr-1* mRNA and in dorsal lateral marginal zone of four-cell stage embryos were shown in (B) and (C), respectively. Both results demonstrated that construct $pmSBE_{1+2}$ exhibit the lowest responsiveness. Transgenic embryos with mutated construct detected with *GFP* probe were shown in (D,E) and (F,G) is a transgenic embryo with p-4223GFP construct with *GFP* probe using as a control. The inset represented endogenous *Xmyf-5* expression in gastrula embryos. Embryos were vegetal view, with dorsal up.

region but failing to form a ring shape (Figs. 5D and E; $pmSBE_1$: 70%, $n = 61$; $pmSBE_2$: 70%, $n = 44$). Taken together, a series of mutation analyses confirmed that the SBEs are both indispensable for conferring Activin/Nodal signals' responsiveness and ventral expression of *myf-5* in *Xenopus* gastrula embryos.

Discussion

The developmental signals such as FGF, Wnt, and BMP are believed to play key roles in muscle progenitor specification by functioning in the positive and negative networks to control *myf-5* and *myoD* activation [3]. Current evidence indicates that the Nodal family of TGF-β-related ligands has been critical regulators of early vertebrate embryogenesis [16]. Mouse embryos homozygous for a null mutation in *nodal* arrest development at early gastrulation and contain little or no embryonic mesoderm. In *Xenopus*, Nodal-related proteins are capable of inducing muscle differentiation during gastrulation [22].

In this report, we demonstrate that Activin/Nodal signals can induce *myf-5* expression both in animal caps and in embryos. The two Smad binding elements (SBEs) identified in *Xmyf-5* promoter are capable of binding to Smad4 protein. These SBEs are both required in conferring reporter gene responsiveness in vitro and mediating reporter gene expression in vivo. To address whether the two SBEs are sufficient to drive reporter gene expression in the ventral marginal zone of gastrula embryos, the construct p-4223/-3798GFP (Fig. 2A) was subjected to transgenesis. No *GFP* positive signal was detected in this transgenic embryo (data not shown), suggesting that the ventral expression of *Xmyf-5* requires additional *cis*-elements. Given that Fast-1 plays a critical role in mediating TGF-β signaling during early developmental stages [30], we had attempted to identify a Fast-1binding site in the *Xmyf-5* promoter but failed. However, we cannot rule out the involvement of other co-factors to participate in the Activin/Nodal signals, as reviewed by Massague and Chen [32]. Nevertheless, our results prove that Activin/Nodal signals are involved in the *Xmyf-5* regulation, especially in the ventral expansion of *Xmyf-5* during gastrulation.

It is generally believed about the ventral marginal zone cells' fate to ventral mesoderm. However, several lines of recent work provided evidence that the ventral marginal zone cells, indeed, contribute to the posterior somites [7,10]. Previous evidence also suggested that Nodal signals are required to position the anterior–posterior axis in mouse and zebrafish [12]. In *Xenopus* gastrulae, although the expression of Nodal-related proteins starts from the dorsal side, their expression domains expand to the ventral side of the embryos during later stage [28,33]. Furthermore, the expression of *derriere*, which is a member of TGF-β superfamily ligands and is involved in the posterior development, starts from the ventral side during gastrulation [34]. Consistent with this idea, the phosphorylation of Smad2, a downstream effector of Activin/Nodal signaling, occurs both in the dorsal and ventral regions from early gastrula (stage 10.5), though it can be detected only in the dorsal side at earlier stage [22]. In the case of *Xmyf-5*, we observed that in transgenic embryos with reporter gene expression in the ventral marginal zone, the reporter gene was also expressed in the posterior somites of tailbud stage embryos (Fig. 3B). Our results, therefore, support the idea that cells of the ventral marginal zone may not develop to ventral cell types. Instead, they may contribute to posterior somites, a dorsal structure, later on.

Acknowledgments

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