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Multi-modal effects of BMP signaling on *Nodal* expression in the lateral plate mesoderm during left–right axis formation in the chick embryo

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ABSTRACT

During development of left–right asymmetry in the vertebrate embryo, *Nodal* plays a central role for determination of left-handedness. Bone morphogenetic protein (BMP) signaling has an important role for regulation of *Nodal* expression, although there is controversy over whether BMP signaling has a positive or negative effect on *Nodal* expression in the chick embryo. As BMP is a morphogen, we speculated that different concentrations might induce different responses in the cells of the lateral plate mesoderm (LPM). To test this hypothesis, we analyzed the effects of various concentrations of BMP4 and NOGGIN on *Nodal* expression in the LPM. We found that the effect on *Nodal* expression varied in a complex fashion with the concentration of BMP. In agreement with previous reports, we found that a high level of BMP signaling induced *Nodal* expression in the LPM, whereas a low level inhibited expression. However, a high intermediate level of BMP signaling was found to suppress *Nodal* expression in the left LPM, whereas a low intermediate level induced *Nodal* expression in the right LPM. Thus, the high and the low intermediate levels of BMP signaling up-regulated *Nodal* expression, but the high intermediate and low levels of BMP signaling down-regulated *Nodal* expression. Next, we sought to identify the mechanisms of this complex regulation of *Nodal* expression by BMP signaling. At the low intermediate level of BMP signaling, regulation depended on a NODAL positive-feedback loop suggesting the possibility of crosstalk between BMP and NODAL signaling. Overexpression of a constitutively active BMP receptor, a constitutively active ACTIVIN/NODAL receptor and SMAD4 indicated that SMAD1 and SMAD2 competed for binding to SMAD4 in the cells of the LPM. *Nodal* regulation by the high and low levels of BMP signaling was dependent on *Cfc* up-regulation or down-regulation, respectively. We propose a model for the variable effects of BMP signaling on *Nodal* expression in which different levels of BMP signaling regulate *Nodal* expression by a balance between BMP-pSMAD1/4 signaling and NODAL-pSMAD2/4 signaling.

Introduction

Nodal plays important roles in patterning of the primary body axis of the vertebrate embryo (Hamada et al., 2002; Shen, 2007; Tabin, 2006). NODAL binds to type I and type II receptors, which signal to the nucleus through SMAD2/SMAD3 and SMAD4 complexes. NODAL can regulate downstream genes only in the presence of co-receptors of the *Cfc* family.

In the vertebrate embryo, *Nodal* plays a central role as a left determinant for patterning the left–right (L–R) axis. In mice, *Nodal* is expressed in perinodal crown cells and is then transferred to the left lateral plate mesoderm (LPM), resulting in asymmetric, left-handed expression of *Nodal* (Hamada et al., 2002; Shiratori and Hamada, 2006). Perinodal expression of *Nodal* is responsible for *Nodal* expression in the left LPM. In the LPM, NODAL activates its own transcription by a positive feedback mechanism in a *Cfc* and *FoxH1* dependent manner. NODAL induces *Lefty-1* and *-2*, which act as negative regulators of NODAL and restrict NODAL activity to the left side of the body. NODAL also induces a left-specific transcription factor *Pitx2* that is implicated in the control of internal organ morphology.

There is increasing evidence that bone morphogenetic proteins (BMPs) also have a role in the regulation of L–R axis formation, although the conclusions of the various reports are not completely consistent. Some studies have reported that BMP signaling has a negative effect on *Nodal* expression (Chang et al., 2000; Kishigami et al., 2004; Rodriguez Esteban et al., 1999; Yokouchi et al., 1999), other studies suggest that it has a positive effect (Fujiwara et al., 2002; Piedra and Ros, 2002; Schlange et al., 2002; Yu et al., 2008). Recently, supportive evidence for a negative role

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for BMP signaling was obtained in the mouse. Phosphorylated SMAD1/5/8 is less abundant in the left LPM and this asymmetric distribution is attributed to BMP inhibition by NOGGIN and Chordin whose expression is enriched in the left LPM (Mine et al., 2008). The conditional deletion of *Smad1* in the LPM confirmed a repressive role for BMP signaling (Furtado et al., 2008). In contrast to the mouse embryo, the role of BMP signaling in the regulation of *Nodal* expression during chick L–R axis formation is still unclear. In chick embryos, BMP signaling has been reported to have a negative influence on *Nodal* expression on the basis that the *Cerberus*/*DAN* family member *Cerberus*/*Caronte* is expressed in the left LPM and induces *Nodal* expression by inhibition of BMP signaling (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). By contrast, a positive role for BMP signaling has been suggested from the observation that application of BMP to the LPM up-regulates *Nodal* expression whereas NOGGIN down-regulates expression (Piedra and Ros, 2002; Schlange et al., 2002). This apparent inconsistency currently precludes a definitive understanding of the role of BMP signaling during L–R axis formation in the chick embryo.

In an attempt to reconcile the contradictory conclusions described above on the role of BMP signaling in the chick embryo, we hypothesized that different concentrations of BMP might have different effects in the regulation of *Nodal* expression. Some morphogens are known to have a dose-dependent effect on the level of gene expression in particular developmental pathways (Affolter and Basler, 2007; Mizutani et al., 2006). BMP is also a morphogen and, therefore, different concentrations might induce different responses in the LPM. In the present study, we examined the responses induced by four levels of BMP signaling on *Nodal* expression in the LPM of chick embryos. We found that BMP signaling modulated *Nodal* expression in a concentration-dependent manner during L–R patterning. Our results suggest that competition between BMP signaling and NODAL signaling regulates *Nodal* expression at intermediate levels of BMP signaling. We propose a model for the variable effects of BMP signaling on *Nodal* expression that accounts for the different effects of different levels of BMP signaling in L–R axis formation in the chick embryo.

Materials and methods

Embryos and experimental manipulations

Embryos were staged using the criteria established by Hamburger and Hamilton (1992) and were cultured using New's method (New, 1955). For bead implantation, Affigel-Blue beads (Bio-Rad) of about 200 μm in diameter were soaked in 1 ng/ml to 10 μg/ml solutions of mouse BMP4 protein (R&D Systems), 25 ng/ml to 10 μg/ml mouse NOGGIN (R&D Systems), or 0.1% BSA/PBS. AG1-X2 beads (Bio-Rad) were soaked in 10 mM SB431542 (Sigma) in DMSO. The beads were implanted as described by Katsu et al. (2012).

Electroporation

The coding sequence of chicken *Cfc* was amplified by reverse transcriptase PCR (RT-PCR) using the primers 5′–GCCATGGTCTGGCGAAAACATGTTAG–3′ and 5′–CGGATCCTCACAACTGCCAGCAGCAAAG–3′; the amplified sequence was cloned into the NcoI-BamHI site of a pSlax21 vector, and then subcloned into the ClaI site of a modified pCAGGS expression vector. The coding sequence of mouse *Smad4* was amplified by RT-PCR using the primers 5′–CCATGGACAATATGTCTATAAC–3′ and 5′–TCAGTCTAAAGGCTGTGGGT–3′; the amplified sequence was cloned into the NcoI-NotI site of a pSlax21 vector, and then subcloned into the ClaI site of a modified pCAGGS expression vector. pCAGGS and pCAGGS-*GFP* were provided by Dr. Takahashi (Nara Institute of Science and Technology). Expression vectors carrying a constitutively active form of the Bmp receptor (pc3-Alk6 (QD)-HA: pc3-*caAlk6*) and the constitutively active form of the Activin/Nodal receptor (pc3-Alk4 (TD)-HA: pc3-*caAlk4*) were provided by Drs. Imamura (Ehime University) and Miyazono (University of Tokyo) (Nakao et al., 1997).

Electroporation was performed as previously described (Granata and Quaderi, 2003). DNA solutions (3–5 mg/ml of pCAGGS for control, 3 mg/ml of pc3-*caAlk4*, 3 mg/ml of pc3-*caAlk6*, 3 mg/ml of pCAGGS-*Cfc*, or 5 mg/ml of pCAGGS-*Smad4*) containing 1 mg/ml pCAGGS-*GFP* vector and 0.1% Fast Green in PBS were placed onto explanted HH4 embryos with a glass capillary. An electric pulse of 5 V, 25 ms was applied three times using a CUY21 electroporator (Tokiwa Science). The positions for gene transfer were selected according to the fate map described by Psychoyos and Stern (1996).

Morpholino oligonucleotides

Morpholino oligonucleotides (MOs) were designed to block translation of Smad1 and Smad2; fluorescein-labeled MOs were obtained from Gene Tools (Philomath, USA). The targeted sequences were as follows: Smad1 MO, 5′–AAACTTGTCACGTTCATGGTGATCC–3′; Smad2 MO, 5′–TGGCAGAATGGATGACATGACTCC–3′. The fluorescein-labeled control morpholino (Gene Tools, Philomath, USA) was used for control experiments. MO solutions (1 mM MO) containing 2 mg/ml pCAGGS vector, 1 mg/ml pCAGGS-*GFP* vector and 0.1% Fast Green in PBS were placed onto explanted HH4 embryos with a glass capillary and electroporated as described above. To assess the efficiency of the Smad1 and Smad2 MOs, embryos were electroporated with MOs and tissue samples of an area approximately $600 \times 600\ \mu m^2$ were excised at HH8 from fluorescein/GFP-positive regions. The explants were homogenized in SDS sample buffer, and subjected to SDS-PAGE and immunoblotting (see below).

Whole-mount in situ hybridization, immunostaining, and TUNEL-labeling

RNA probes for *Shh* and *Nodal* were prepared as described by Levin et al. (1995). A 504 bp fragment of chicken *Cfc* was obtained by RT-PCR using the primers 5′–TCCGTGCCTGTCTTGGTACTGT–3′ and 5′–AGTCGCCATGGATGATGCTG–3′. Whole-mount in situ hybridization was carried out as described by Katsu et al. (2012) using digoxigenin-labeled RNA probes.

Whole-mount immunostaining was carried out using the method of Faure et al. (2002) with an anti-phosphorylated SMAD1/5 antibody (1:100, Cell Signaling, 41D10, #9516). Peroxidase conjugated goat anti-rabbit IgG (1:300, Vector) was used as the secondary antibody. The fluorescent signal was developed using the tyramide signal amplification Plus system (PerkinElmer).

TUNEL-labeling of whole mount preparations was performed using a previously described method (Noro et al., 2011) except that proteinase K was used at 1 μg/ml for 15 min and the TUNEL reaction was performed using the In Situ Cell Death Detection Kit, TMR red (Roche). We counted TUNEL-positive nuclei in an area approximately $600 \times 600\ \mu m^2$ around the implanted bead and a similarly sized area on the contralateral side.