Cooperative action of Sox9, Snail2 and PKA signaling in early neural crest development

Daisuke Sakai¹, Takashi Suzuki¹, Noriko Osumi¹ and Yoshio Wakamatsu²,*

In neural crest formation, transcription factors, such as group E Sox and Snail1/2 (Snail2) regulate subsequent epithelial-mesenchymal transition (EMT) and migration. In particular, Sox9 has a strong effect on neural crest formation, EMT and differentiation of crest-derived cartilages in the cranium. It remains unclear, however, how Sox9 functions in these events, and how Sox9 activity is regulated. In this study, our gain-of-function and loss-of-function experiments reveal that Sox9 directly activates the Snail2 promoter, although Snail family proteins have been known as a repressor. Consistently, Sox9 directly activates the Snail2 promoter in synergy with, and through a direct binding to, Snail2. Finally, functions of these transcription factors in neural crest cells are enhanced by PKA signaling.

KEY WORDS: Neural crest, Sox9, Snail, Slug, Snail2, PKA, BMP, EMT, Quail

INTRODUCTION

Neural crest is formed at the boundary of neural plate and non-neural ectoderm in vertebrate embryos, and crest-derived cells subsequently migrate to give rise to various tissues, including neurons, glial cells, melanocytes and cranial mesenchymal tissues (Le Douarin and Kalcheim, 1999). The first sign of neural crest formation is expression of a set of transcription factors in the neural fold, followed by epithelial-mesenchymal transition (EMT) (for reviews, see Meulemans and Bronner-Fraser, 2004; Sakai and Wakamatsu, 2005; Kalcheim and Burstyn-Cohen, 2005).

Bone morphogenetic protein (BMP) signaling is important for the neural crest induction. For example, BMP2/4/7 can induce neural crest markers, such as Snail2 (previously known as Slug) and HNK-1 in neural plate explants (Liem et al., 1995; Liem et al., 1997; Liu and Jessell, 1998; Marchant et al., 1998; García-Castro et al., 2002; Wakamatsu et al., 2004a) and the BMP signal is required for the crest induction in head neural folds of avian embryos (Endo et al., 2002). Recently, we showed that Notch signaling regulates Bmp4 expression and thereby crest formation (Endo et al., 2002; Endo et al., 2003), but other signals, such as Wnt, FGF and retinoids also appear to be involved in neural crest formation in various vertebrate species (LaBonne and Bronner-Fraser, 1998; García-Castro et al., 2002; Villanueva et al., 2002; Monsono-Burq et al., 2003; Bastidas et al., 2004).

Under the influence of inducing signals, expression of crest-specific transcription factors will be promoted to regulate the following events in the crest development. Snail and Snail2 [see Barrallo-Gimeno and Nieto (Barrallo-Gimeno and Nieto, 2005) for nomenclature] genes, both of which encode Zn-finger-type transcription factors, have been shown to be involved in neural crest formation and subsequent EMT (Nieto et al., 1994; LaBonne and Bronner-Fraser, 2000; del Barrio and Nieto, 2002). Other transcription factor genes, such as Msx1, Foxd3 and group E Sox genes (Sox8, Sox9 and Sox10), are also involved in early events of neural crest development (Kos et al., 2001; Sasai et al., 2001; Dottori et al., 2001; Spokony et al., 2002; Cheung and Briscoe, 2003; Honore et al., 2003; Tribulo et al., 2003; Lee et al., 2004; Cheung et al., 2005; McKeown et al., 2005). By contrast, Sox2, a member of the group B1 Sox genes, is expressed in the neural plate and inhibits neural crest formation and EMT (Wakamatsu et al., 2004a).

Among these transcription factor genes, Sox9 appears to have a central role in neural crest formation and subsequent EMT. In mice carrying a mutation in Sox9, the number of crest cells is severely decreased (Cheung et al., 2005). Both in Xenopus and chicken embryos, Sox9 overexpression promotes an increase of crest-like cells (Spokony et al., 2002; Cheung and Briscoe, 2003; McKeown et al., 2005).

Despite rapid progress in this field to identify genes involved in neural crest development, hierarchical relationship of such genes is largely elusive. In particular, our knowledge on the regulation of crest-specific genes at transcriptional level is extremely limited. In our previous study, we analyzed the regulation of the promoter activity of chick Snail2, and revealed that Snail2 is directly regulated by BMP and Wnt signals (Sakai et al., 2005). In this study, we show: (1) that Sox9 function is required for BMP-mediated Snail2 induction and EMT; (2) that Sox9 directly activates the Snail2 promoter in synergy through a physical interaction with Snail2 protein; and (3) that cAMP-dependent protein kinase (PKA) signal facilitates Snail2 induction and EMT, partly by promoting Sox9 and Snail2 function. Our results provide significant steps forward in understanding the regulation of early neural crest development.

MATERIALS AND METHODS

Experimental animals

Japanese quail (Coturnix coturnix japonica) eggs were obtained from Sendai Jun-ran, Sendai. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) (HH stage).

Expression vectors

The coding sequences of quail Snail2, Sox9 and PKIα were PCR-amplified from oligo(dT)-primed E2 embryonic cDNA, and inserted into pyDF30 for N-terminal FLAG-tagging and expression. Sox9 was also subcloned into pyDF-HA for N-terminal-HA tagging. The zinc-finger domain of Snail2 cDNA was further PCR amplified, and fused to the activation domain of VP16 and
repression domain of Engrailed2 (a gift from Dr H. Nakamura) (Matsunaga et al., 2000). To generate a repressor form of Sox9, the C-terminal sequence downstream of its HMG-domain was removed, Engrailed2 repressor domain was connected (pyDF30 FLAG-Sox9-En), and to facilitate nuclear localization, a nuclear translocation signal taken from pDsRed2-nuc (Clontech) was inserted between Sox9 and Engrailed2 sequences (pyDF30-FLAG-Sox9-En-nuc). Dominant-negative action of this Sox9 mutant over wild type was confirmed by a Luciferase assay with Snail2 D3.0 reporter in NIH3T3 cells (not shown). Expression vectors of chicken Sox10 (Cheng et al., 2000), chicken Foxd3 (Kos et al., 2001) and mouse Msx1 were kindly provided by Drs P. Scotting, C. Erickson and K. Tamura, respectively. CA-PKA and Sox9\textsuperscript{min2} were generous gifts from Dr B. de Crombrugge (Huang et al., 2000). pEGFP-N1 was purchased from Clontech.

**In situ hybridization**

Whole-mount in situ hybridization was performed as described previously (Wakamatsu and Weston, 1997). Quail Snail2 cDNA for cRNA probe was described previously (Wakamatsu et al., 2004b). Quail Sox9 was PCR-amplified from oligo (dT)-primed E2 embryo cDNA. Chicken cDNAs of Sox10 (Cheng et al., 2000), Foxd3 (Kos et al., 2001), Msx1 and Msx2 were kind gift from Drs P. Scotting, C. Erickson and K. Tamura, respectively. Luciferase assay

The upstream sequences of Snail2 subcloned into pGL3-basic (Amersham-Pharmacia) Luciferase assay vector were described previously (Sakai et al., 2005). NIH3T3 cells were transfected with a Luciferase reporter and effector plasmid DNAs with LipofectAMINE Plus reagent (Invitrogen). pRL-TK was always co-transfected to normalize for transfection efficiency (Dual-Luciferase Assay System; Promega). Cell lysates were prepared for Luciferase activity after 24 hours of culture with PicaGene Dual kit (Toyo Ink). To activate PKA signal, 1 mM of CAMP analog 8-bromo-cAMP (Calbiochem) was added in culture. Activation of the signal was confirmed by the activation of pCRE-luciferase reporter (Clontech).

The Luciferase reporter constructs were also electroporated to medial neural plate of stage 6 quail embryo and neural plate explants were taken from embryos as described below. pRL-SV40 (Amersham-Pharmacia) was co-electroporated to normalize for electroporation efficiency. Neural plate explants were cultured as described below (see also Wakamatsu et al., 2004a). Cells were collected and Luciferase activity was assayed after 20 hours of culture.

**Electro-mobility shift assay (EMSA)**

Recombinant GST-Snail2 fusion protein was purified from bacterial lysate carrying pET11GST-Snail2 expression vector. Sox9 proteins were produced by an in vitro transcription/translation system using TNT T7 Quick Master mix (Promega). The DNA-binding mix contained proteins and 32P-labeled double-strand oligonucleotide in a binding buffer [10 mM HEPES (pH 7.9), 100 mM NaCl, 1 mM DTT, 5 mM EDTA, 5% glycerol and 150 ng of pol (dI-C)]. The binding reaction was performed with or without unlabeled competitor DNA, incubated at 30°C for 30 minutes. After incubation, reaction mixtures were subjected to electrophoresis and signals were detected by autoradiography. The nucleotide sequence of the probes and competitors were as follows: E-box2, AACCCTCCGTGACA-TGACTTGGC; mE-box2, AAACCCCTCCAGCACGGTGGC; mE-box2(1), GAAGAGAAAAACCCACACCCTCTT; mE-box2(2), CACCT- GACTGCGGTATTCCG; mE-box2(3), ACAACCCCTGCACT- GACTTGGC; mE-box2(4), AACCAACTCGACTGGTGGC; mE-box2(5), ACCCCCCAGTCCACTGACTTGGC.

**Co-immunoprecipitation assay**

Sox9 full coding sequence, N-terminal deletion (Sox9\textsuperscript{ΔN}, carrying amino acids 253-494) and C-terminal deletion (Sox9\textsuperscript{ΔC}, carrying amino acids 1-253) were subcloned into pET11 GST for N-terminal GST-tagging, and transformed E. coli BL21(DE3) strain (Fig. 5A). Recombinant GST-Snail2 proteins were purified from whole cell lysates by B-PER GST Fusion Protein Purification kit (PIERCE).

To detect the interaction of GST-Snail2 fusion proteins and HA-Sox9 proteins, immunoprecipitation was performed using ProFound Mammalian HA-tag IP/Co-IP kit (PIERCE). After 24 hours of culture, COS7 cells transfected with HA-Sox9 constructs were lysed in M-PER buffer (PIERCE) and GST-Snail2 fusion proteins were added into the cell lysate, and incubated for 24 hours at 4°C with gentle agitation. Protein complexes in cell extracts were precipitated with immobilized anti-HA antibody agarose beads (PIERCE). Precipitated complexes were eluted by elution buffer and denatured in SDS sample buffer at 95°C for 5 minutes. Co-precipitated proteins were separated by SDS-PAGE, and detected by western blotting using anti-HA (Roche) and anti-GST (Santa Cruz) antibodies.

**Neural plate explant culture**

Cultures of neural plate explants were performed as described previously (Wakamatsu et al., 2004a; Sakai et al., 2005). In brief, intermediate fragments of the neural plate at the level of fore- and midbrain were surgically dissected with a tungsten needle along with underlying mesoderm and endoderm. To remove the mesoderm and the endoderm, the dissected tissues were treated with Pancreatin (Gibco). The isolated neural plates were cultured in F12-based medium containing 3% FCS on fibronectin (Sigma)-coated dishes. N2-supplement (1/100 dilution, Invitrogen) and recombinant human BMP4 (20 ng/ml, R&D systems) were added in culture to induce neural crest formation. To inhibit PKA signal, 1 or 10 μM of H89 (Biomol) was added in culture. To obtain consistent results, more than 15 explants were examined in three independent experiments of each condition.

To knockdown Sox9 expression in the explants, double-strand RNAs (dsRNA) for quail Sox9 (sense strand: GGAAAGGCGACGAGCAAA) as well as a mutated control dsRNA (GGATGCGACCCTGACAAA) were generated (Takara) and the dsRNAs (10 μM/PBS) were electroporated to stage 6-7 quail embryos, as indicated below.

To compare the level of Snail2 mRNA expression in the neural plate explants, total RNA was prepared by RNeasy kit (Qiagen) and cDNA was generated with SuperscriptII (Invitrogen). The number of PCR cycles required for a linear amplification of Snail2 cDNA was determined, and amplified bands of Snail2 were normalized with those of GAPDH and were semi-quantified with NIHimage software. The primers used were: Snail2-F, CTCCCCCTCCTGTACGAC; Snail2-R, CTTTCTGAAC- GGCTTGATC; GAPDH-F, GTGAAAGTCCGAGTCAAGC; GAPDH-R, AGTTGTGGTACGATGCA.

**Whole embryo culture and gene transfer**

The whole embryo culture in combination with electroporation was described previously (Endo et al., 2002; Sakai et al., 2005). In brief, before electroporation, the embryos adhering to collagen-coated membranes were set on a chamber with a 2 mm2 positive electrode (Unique Medical Imada). A tungsten needle was used as the negative electrode. DNA solution (1.2 μl of 5 μg/μl in PBS containing 0.025% Fast Green) was placed on the right ectoderm of the embryo. The condition of electroporation was 3×7V for 25 ms at 200 ms interval. For misexpression studies, pEGFP-N1 and another expression vector were mixed at 1:1.

**Antibodies and immunological staining**

62.1E6 anti-Snail2 (mouse IgG1; DSHB) (Liem et al., 1995) antibody was used as described previously (Wakamatsu et al., 2004a; Sakai et al., 2005). M2 anti-FLAG (mouse IgG1, Sigma), anti-β-galactosidase (rabbit polyclonal, Cappel), anti-HA (rabbit IgG, Roche), anti-phospho-CREB (rabbit IgG, Cell Signaling) and anti-phospho-Sox9 (rabbit IgG, Abcam) antibodies were commercially obtained. Anti-chicken Sox9 was a kind gift from Dr R. Lovell-Badge (Morsa da Silva et al., 1996). Fluorochrome-conjugated secondary antibodies were purchased from Jackson, Phalloidin conjugated with Texas Red-X or Oregon Green were obtained from Molecular Probes.

Immunological staining on sections and cultured cells was performed as described previously (Wakamatsu et al., 1993; Wakamatsu et al., 1997). Sections treated with antibodies were also exposed to DAPI (Sigma) to visualize nuclei. Whole-mount detection of phospho-CREB was performed essentially as described (Endo et al., 2002).
RESULTS
Comparative analysis of expression of neural crest-specific transcription factors

In previous studies, expression of various transcription factors in the neural crest has been described. However, expression patterns have not been compared systematically, particularly at the cranial level. Because we have documented the expression of Snail2 in detail at the cranial level of quail embryos (Endo et al., 2002; Wakamatsu et al., 2004a), we sagittally bisected Hamburger-Hamilton (HH) stage 6-9 (Hamburger and Hamilton, 1951) quail embryos, and examined expression of Snail1, Sox9, Sox10, Foxd3, Msx1 and Msx2 in the left half, and compared it with the expression of Snail2 in the right half, in whole-mount preparations (Fig. 1, Table 1). We confirmed, as previously shown (Sefton et al., 1998), that no Snail1 expression was observed in the head neural folds or emigrating crest cells (data not shown). As previously described (Endo et al., 2002), Snail2 mRNA expression was first detected in the head neural folds around the first somite stage (HH stage 6.5). Although Sox9 expression was already observed in the midline tissue at HH stage 6, it was not detectable in the neural crest domain until HH stage 6.5, and increased at later developmental stages. Msx1 expression in the boundary between neural plate and non-neural ectoderm was already observed at HH stage 6, and the expression in the neural fold was continuously observed at later stages. Msx2 expression in the neural folds was observed later than Snail2 and Msx1 expression, and neural fold expression in the fore- to midbrain level was observed only at HH stage 8 (four somite stage) and following stages. Foxd3 expression in the head neural fold was faintly observed at HH stage 7 (one to two somites), while strong expression was already observed in the anterior-medial neural tissues. Sox10 expression was barely detectable at HH stage 8, and strong expression was observed at stage 9 (seven somites) in the cranial neural folds and in delaminated neural crest cells.

Sox9 expression is required for neural crest formation

As Sox9 was expressed in the cranial neural folds as early as Snail2 (Fig. 1), and as previous studies in chick trunk and Xenopus head neural folds (Spokony et al., 2002; Cheung and Briscoe, 2003; Cheung et al., 2005; McKeown et al., 2005) showed an involvement of Sox9 in neural crest formation and subsequent EMT, we focused on the function of Sox9 in cranial neural crest development in avian embryos.

First, we examined Sox9 protein expression in cultured neural plate explants taken from the fore and midbrain levels. In previous studies, we have shown that a treatment of the explants with BMP4 can effectively induce expression of Snail2 and subsequent EMT (see Materials and methods, see also Fig. 2A, Fig. 6A) (Wakamatsu et al., 2004a; Sakai et al., 2005). Under these conditions, Sox9 expression was also induced effectively (Fig. 2A), although no Sox9 immunoreactivity was detected in cultures without BMP4 in the medium (Fig. 2A). Next, expression vectors of either Snail2 or Sox9 were co-transfected with a GFP expression vector into the neural plate explants, and the transfected explants were cultured in the absence of BMP4 (Fig. 2B,C). Although Snail2 misexpression induced neither Sox9 expression nor EMT (Fig. 2B), strong expression of endogenous Snail2 was induced by the transfection of Sox9 (Fig. 2C). Sox9-transfected cells dispersed on culture dishes and fibroblastic appearance of actin stress fibers lacking junctional actin bundles indicated a promotion of EMT (Fig. 2C). To study Sox9 function in vivo, an expression vector of En-fusion of Sox9 (Sox9-En-nuc, constitutive

Fig. 1. Comparative analysis of the expression of transcription factors, such as Sox9, Msx1, Msx2, Foxd3, Sox10 and Snail2, from a dorsal view. Stage 6-9 embryos were sagittally bisected, and the right halves were hybridized with Snail2 probe, while the left halves were hybridized with other probes for close comparison. Arrowheads indicate expression in the head neural folds. Asterisks indicate expression of Sox9 and Foxd3 in the midline tissues.
Table 1. The onset and the level of gene expression in neural fold/neural crest

<table>
<thead>
<tr>
<th>Gene</th>
<th>HH stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Snail2</td>
<td>-</td>
</tr>
<tr>
<td>Sox9</td>
<td>-</td>
</tr>
<tr>
<td>Msx1</td>
<td>+</td>
</tr>
<tr>
<td>Msx2</td>
<td>-</td>
</tr>
<tr>
<td>Foxd3</td>
<td>-</td>
</tr>
<tr>
<td>Sox10</td>
<td>-</td>
</tr>
</tbody>
</table>

Snail2 auto-activation

In a previous study, we have isolated a promoter sequence of chicken Snail2 (Sakai et al., 2005). Whereas Smad1, a BMP signal transducer, could directly bind to the Snail2 promoter sequence and activate the promoter, the promoter lacking Smad-binding sites still responded to the BMP signal to some extent (Sakai et al., 2005). This suggested that BMP signal could also indirectly activate the Snail2 promoter via other transcription factor(s). In Xenopus, exogenous Snail2 has been shown to induce endogenous Snail2 expression (LaBonne and Bronner-Fraser, 2000). We thus tested auto-regulation by Snail2 in the NIH3T3 mouse fibroblast cell line. Transfection of Snail2 stimulated the Snail2 promoter-Luciferase reporter (D0.1), which contains a conserved E-box motif (see Sakai et al., 2005), in a dose-dependent fashion (Fig. 2A), suggesting that Snail2 activated its own promoter either directly or indirectly.

As previous studies (Inukai et al., 1999; Hemavathy et al., 2000) have shown that Snail2 preferentially binds to an E-box-like sequence, and as two E-box have been found in the Snail2 promoter (Sakai et al., 2005), we reasoned that Snail2 might directly bind to one of the E-box sequences to activate the promoter. Since D0.1 reporter lacked the E-box1, which was recognized by MyoD (Zhao et al., 2002), we focused on E-box2, which is conserved between Snail2 orthologs of several species (Sakai et al., 2005). As we anticipated, a direct binding of Snail2 to the E-box2 was detected by EMSA (Fig. 3B). Consistently, a reporter carrying a mutation in the E-box2 (Em2) showed little response to Snail2 expression in a Luciferase assay (Fig. 3C).

As Snail family proteins have been considered to be repressors (for a review, see Nieto, 2002), and because in Xenopus, Snail2 appears to function as a repressor in crest development (LaBonne and Bronner-Fraser, 2000), our observation that Snail2 directly activated the Snail2 promoter appeared paradoxical. However, when 3T3 cells were transfected with an activator form of Snail2, VP16-Snail2, the D0.1 reporter was strongly activated, while transfection of a repressor form, En-Snail2, showed little activation (Fig. 3D). Taken together, these results confirmed the idea that Snail2 can directly activate its own promoter. This auto-activation alone seemed supportive only for the expression of Snail2, as Snail2 transfection into the neural plate explants increased the expression of endogenous Snail2 mRNA weakly and transiently (Fig. 3E).

Next, we performed En-Snail2 transfection into the ectoderm of cultured quail embryo, and this also reduced the expression of endogenous Snail2 (6/6 cases, Fig. 3F), indicating the requirement of Snail2 as an activator. It should be emphasized, however, that transfection of VP16-Snail2 into cultured embryos decreased the endogenous Snail2 expression (6/6 cases, Fig. 3G), suggesting the requirement of Snail2 as a repressor. Similarly, transfection of VP16-Snail2 into neural plate explants inhibited the induction of endogenous Snail2 expression and EMT by BMP4 added in the culture medium (Fig. 3G). Therefore, Snail2 could act both as an activator and a repressor, probably dependent on the target genes, and both activator and repressor functions of Snail2 seemed to be required for neural crest formation and subsequent EMT in avian ectoderm.

Sox9 synergistically activates the Snail2 promoter

We considered the possible involvement of other transcription factors for Snail2 activation. As mentioned in the Introduction, both Foxd3 and Sox9 have been shown to be involved in neural crest formation both in Xenopus and chick (Kos et al., 2001; Sasai et al., 2001; Dottori et al., 2001; Spokony et al., 2002; Cheung and Briscoe, 2003; Cheung et al., 2005; McKeown et al., 2005). In Xenopus, Msx1 and Sox10 have also been shown to be involved in this process (Tribulo et al., 2003; Honore et al., 2003), and overexpression of Sox10 induced ectopic EMT in the chick neural tube (McKeown et al., 2005). Thus, we used the Luciferase assay in neural plate explants to test the ability of transcription factors (Mxs1, Foxd3, Sox9, and Sox10) to activate the D1.2 Snail2 reporter construct, which contains all the cis-regulatory elements required for the expression in the neural crest (Sakai et al., 2005). We found that only Sox9 could activate the reporter (Fig. 4A).

To determine if Sox9 directly bound to the Snail2 promoter, EMSA was performed (Fig. 4B). A clear binding of Sox9 was observed when a probe, including the flanking sequence of the E-box2 was used. Competitions with mutated sequences indicated that the C-rich sequence adjacent to the E-box2 was important for the Sox9 binding (Fig. 4B). Accordingly, a Snail2 promoter-Luciferase reporter containing a mutation in this sequence (Em3) showed little response to Sox9 expression (Fig. 4C).

Interestingly, although Sox9 alone could activate the Snail2 promoter only moderately, co-transfection of Sox9 and Snail2 synergistically activated the D0.1 reporter to higher levels (Fig. 4D). The synergistic activation of the Snail2 promoter by Sox9 and Snail2, and closely located binding sites of these factors in the promoter sequence is consistent with a physical
interaction of these proteins. Co-immunoprecipitation analysis revealed a clear association of Sox9 and Snail2 (Fig. 5). Deletion mutagenesis of both Sox9 and Snail2 showed that their interaction was mediated through the N-terminal Sox9 sequence, including the HMG-box, and the C-terminal Snail2, containing the Zn fingers (Fig. 5).

**Protein kinase A signal enhances Sox9 and Snail2 activity**

Although Sox9 expression could be detected strongly in the premigratory neural crest cells both at the cranial and the trunk levels (see also Cheung and Briscoe, 2003), Sox9 expression was also detected in more ventral neural tissues of brain and neural tube (see Fig. 1; data not shown). In previous studies, in ovo electroporation of Sox9 expression vector into the ventral neural tube did not effectively induce EMT, while co-transfection of Sox9 and Snail2 promoted ectopic EMT more efficiently (Cheung and Briscoe, 2003; Cheung et al., 2005) (but see McKeown et al., 2005). We also observed that misexpression of Bmp4 in vivo expanded Snail2 expression in the neural fold, but more ventral neural plate cells rarely expressed Snail2 (Endo et al., 2002). These observations suggested that the activities of these crest factors were spatially modulated by other signals, possibly provided by surrounding tissues in vivo.

As a candidate of signals that modulates Sox9 function in neural crest formation and EMT, we focused on cAMP-dependent protein kinase A (PKA)-mediated signal, because Sox9 phosphorylation by PKA has been shown to enhance Sox9 function in the transcriptional

![Fig. 2. Sox9 is required for Snail2 induction downstream of BMP signal.](image)

(A) BMP4 treatment (lower panels) of the neural plate explants effectively induces Sox9 expression and cell dispersal. (B) Neural plate explants were co-transfected with Snail2 and GFP expression vectors, and stained with anti-Sox9 antibody (upper panels) or phalloidin (lower panels). Snail2 misexpression fails to induce either Sox9 protein expression or epithelial-mesenchymal transition (EMT). (C) Sox9 transfection strongly induces Snail2 expression in neural plate explants and subsequent EMT indicated by an extensive cell dispersal, in the absence of BMP4. Insets indicate higher magnification, showing typical morphology of Sox9-transfected cells. (D) Electroporation of GFP and Sox9-En-nuc expression constructs was performed on quail embryos at stage 5, and the embryos were cultured for 7 hours. Misexpression of Sox-En-nuc expression vector reduces the Snail2 mRNA expression (arrowheads), compared with the untransfected side, or a control embryo transfected with GFP alone. (E) Sox9 is required for Snail2 induction by BMP4. Neural plate explants, transfected with double-stranded RNA corresponding to Sox9 sequence (Sox9 dsRNA), show little expression of Snail2 or cell dispersal, in the presence of BMP4. Transfection of mutated dsRNA (Sox9mut dsRNA) has no effect (Sox9+Sox9 dsRNA) and the effect of Sox9 dsRNA is cancelled by a co-transfection of Sox9 (Sox10+Sox9 dsRNA), while co-transfection of Sox10 fails to cancel the effect of Sox9 dsRNA.
Thus, although the PKA activity is required for the induction of Sox9m1m2 explants transfected with the presence of BMP4 and H89 failed to express Snail2 (Fig. 6A), induce EMT (Fig. 6C), suggesting the PKA-phosphorylation was approached. Thus, a mutant version of inhibited other kinases non-specifically, we also took a different manner (Fig. 6A,B). However, a high dose of H89 might have the promotion of EMT by BMP4 and Sox9 in 3T3 cells as expected (data not shown). H89 effectively inhibited revealed that Sox9 phosphorylation by PKA was inhibited by H89 in 3T3 cells, suggesting multiple sites of action for the PKA signal.

To test the importance of PKA signal for neural crest formation, we next observed the in vivo distribution of phosphorylated CREB protein, which is expressed ubiquitously and is activated by PKA-mediated phosphorylation (for a review, see Shaywitz and Greenberg, 1999). Although phospho-CREB could broadly be observed, slightly higher levels of phosphorylation were observed in the neural folds of stage 7-8 embryos (Fig. 7A,B), at least indicating PKA activity in the corresponding region. Next, to activate the PKA signal in vivo, constitutively active form of PKA (CA-PKA) (Huang et al., 2000) was transfected into the ectoderm inhibits the expression of endogenous Snail2 protein in cultured embryos (arrowheads). GFP-fluorescence indicates the transfected area. Compare with the un-transfected, left neural folds. As the anti-Snail2 antibody recognizes the N-terminal sequence, it detects only endogenous Snail2 protein. (G) Transfection of VP16-Snail2 inhibits the induction of endogenous Snail2 expression in neural plate explants treated with BMP4.

Fig. 3. Snail2 activates its own promoter as a transcriptional activator. (A) A dose-dependent activation of D0.1-Luciferase reporter gene by Snail2 in NIH3T3 cells. Different amounts of Snail2 expression vector were co-transfected with the Snail2 promoter-Luciferase. (B) Snail2 protein binds to the E-Box2 sequence. Isotope-labeled probe containing the E-Box2 was incubated with Snail2 protein, and subjected to EMSA. The shifted band is diminished by a preincubation of the Snail2 protein with cold wild-type competitor, but persists by a preincubation with E-Box2-mutated competitor. (C) E-box2 is required for the Snail2 promoter activity by Snail2. When E-box2 in the D0.1 reporter is mutated (Em2), activation level of the promoter by Snail2 (+) is significantly decreased, compared with the wild type. (D) Snail2 acts as a transcriptional activator on the Snail2 promoter. An expression vector of VP16 activation domain and Snail2 zinc-finger motifs (VP) strongly activates the D0.1 promoter, while an expression vector of Engrailed2 repression domain and Snail2 zinc-finger fusion (En) has no significant effect on the Snail2 promoter activity. Wt, wild type Snail2. (E) Semi-quantitative RT-PCR analysis of endogenous Snail2 expression in neural plate explants, transfected with VP16-Snail2 and cultured without BMP4. Expression level is normalized with the value of amplified GAPDH. Result obtained from explants cultured for 18 hours with BMP4 are also indicated. (F) Transfection of En-Snail2 and VP16-Snail2 into ectoderm inhibits the expression of endogenous Snail2 protein in cultured embryos (arrowheads). GFP-fluorescence indicates the transfected area. Compare with the un-transfected, left neural folds. As the anti-Snail2 antibody recognizes the N-terminal sequence, it detects only endogenous Snail2 protein. (G) Transfection of VP16-Snail2 inhibits the induction of endogenous Snail2 expression in neural plate explants treated with BMP4.
only with GFP (6/6). These results both in vivo and in vitro indicated the importance of PKA signal in Snail2 expression and subsequent EMT.

As described above, some aspect of Sox9 function could depend on the PKA signal, but the induction of endogenous Snail2 expression did not appear to rely on the signal (see above). Thus, we tested whether PKA activation could affect the transcriptional activation of the Snail2 promoter (Fig. 9). Neither addition of cAMP analog in the culture medium nor co-transfection of constitutively-active PKA (CA-PKA) significantly increased the Sox9-mediated activation of the Snail2 promoter. Consistently, mutations in the PKA-phosphorylation sites in Sox9 did not affect the transcriptional activation of the promoter (Fig. 9). Interestingly, the transcriptional activation of the Snail2 reporter by Snail2 protein was enhanced both by an addition of cAMP analog and by co-transfection of CA-PKA (Fig. 9), although the mechanisms by which PKA signal activates Snail2 function remain to be studied, further suggesting the importance of PKA signal in the early neural crest development.

**DISCUSSION**

Sox9 activates Snail2 expression and neural crest formation

As mentioned above, Sox9 and other groupE Sox genes such as Sox10 appear to be important for neural crest development (Spokony et al., 2002; Cheung and Briscoe, 2003; Honore et al., 2003; Cheung et al., 2005; Lee et al., 2004; McKeown et al., 2005) (this study). Among those, Sox9 is expressed earlier than other family members (this study) (see Cheung and Briscoe, 2003; McKeown et al., 2005).
In this study, we show that Sox9 directly activates the Snail2 promoter. Furthermore, we show that Sox9 is a highly potent inducer of Snail2 expression and EMT in transfected neural plate explants, and that Sox9 function is required for the process. As BMP4 effectively induce Sox9 expression, and as Snail2 activity is necessary (Nieto et al., 1994) (this study) but not sufficient for EMT (del Barrio and Nieto, 2002) (this study), Sox9 is likely to mediate, at least in part, BMP signal to promote neural crest formation (Fig. 10), while BMP signal also activates Snail2 promoter directly (Fig. 10) (Sakai et al., 2005).

How Sox9 promotes neural crest development is largely unknown. Sox9 is expressed in many tissues, and probably regulates distinct sets of genes, depending on the cellular context. For example, Sox9 is involved in cartilage differentiation and directly upregulates Col2a1 (Lefebvre et al., 1997). Other than Snail2, however, there are no crest genes directly regulated by Sox9 known so far. In a previous study, we have shown that Sox2, a group B1 Sox gene, is expressed in the neural plate, and inhibits neural crest formation (Wakamatsu et al., 2004a). Thus, one possible way that Sox9 (and other group E Sox genes) might promote neural crest formation is to interfere with Sox2 function by competing for the binding sites. However, DNA sequences recognized by Sox9 are highly divergent, in contrast to the faithful binding of Sox2 and related group B1 Sox proteins to the Sox consensus binding sequence (Kamachi et al., 1999) (this study). In fact, unlike Sox9, Sox2 does not affect Snail2 promoter activity, at least in NIH3T3 cells (D.S. and Y.W., unpublished). In any case, it is essential to find more Sox9 targets to further understand the Sox9 function in early neural crest development.

Snail2 as a transcriptional activator

In this study, we show that Snail2 activates its own promoter by a direct binding to E-box2. To our knowledge, this is the first case of activation of a target gene by Snail family proteins, although a potential transcriptional activation domain has been suggested in artificial assay system (Hemavathy et al., 2000). This inference appears superficially to conflict with previous reports, showing that Snail2 is involved in a neural crest formation as a transcriptional repressor (LaBonne and Bronner-Fraser, 2000; del Barrio and Nieto, 2002). However, misexpression of VP16-Snail2 in neural plate explants also activated endogenous Snail2. Moreover, consistent with previous reports, VP16-Snail2 strongly inhibited BMP-induced Snail2 expression and EMT. Thus, Snail2 may act as an activator on its own promoter, but it must also act on other target genes as a repressor. The function of Snail2 as an activator probably depends on a partner protein(s), as mutations in the Sox9 binding sequence in the Snail2 promoter decreased Snail2 mediated activation of the
promoter in 3T3 cells even when Sox9 was not co-transfected (D.S. and Y.W., unpublished). In any case, the next challenge will be to identify target genes and partner proteins of Snail2 and to study the mechanism by which Snail2 is converted from a repressor to an activator, or vice versa.

Cooperative action of Snail2 and Sox9

Our study revealed that the Snail2 auto-activation is enhanced by an interaction of Snail2 and Sox9. Although Sox family protein has been shown to require co-factors for transcriptional activation, the combination of Snail2 and Sox9 is unique, as reported examples for Sox partner are mostly homeobox-containing proteins, such as Oct3/4, Pax6 and Brn2 (for a review, see Kondoh et al., 2004). Nevertheless, the partnership of Sox9 and Snail2 is in line with a previous report, showing that co-transfection of Sox9 and Snail2 effectively induced ectopic EMT in the neural tube (Cheung et al., 2005). There will probably be more target genes of the Sox9-Snail2 complex, considering the strong effect of the combination.

Modulator of neural crest-inducing signals

Although many inducing signals such as BMP and Wnt for neural crest formation have been suggested, there are few reports for inhibitory signals and factors. However, as the initial patterning of the embryonic ectoderm is mediated not only by the inducing signals such as BMP, Wnt and FGF proteins, but also by inhibitory factors such as Noggin, it is likely that similar mechanisms may be involved in the spatially restricted formation of the neural crest. In fact, emerging evidences indicate relatively broad tissue distribution of inducing signal inputs, such as the localization of phospho-Smad1 for BMP signal (Faure et al., 2000; Faure et al., 2002; Sakai et al., 2005), and expression of reporter gene containing Lef/Tcf binding sites for canonical Wnt signal (Sakai et al., 2005), compared with the relatively narrow domain of neural crest marker expression. This can be explained by the level of signal inputs, but such pattern can also be established by the opposing effects of facilitating and inhibitory signals.

In this study, we indicate that the PKA signal facilitates Sox9/Snail2 function to promote crest formation and EMT (Fig. 10). It remains unclear, however, if spatially regulated PKA signal contributes restricted neural crest formation in the embryonic ectoderm. Based on the distribution of phospho-CREB immunoreactivity, PKA signal activity may be broad in the early avian embryos, thus PKA signal would function as a permissive signal. However, because there is no method available to detect PKA activity directly in vivo, and as transfection of CA-PKA ectopically induces Snail2 expression, spatially restricted PKA activity may still contribute for the patterned induction of the neural crest and EMT. It is worth mentioning that, because the CA-PKA transfection did not posteriorly expand the fore- to midbrain domain, which produces robust Snail2-positive crest cells compared with more posterior axial level, the ectopic induction of Snail2 does not appear to be secondary to the axial shift. Although regulator(s) of PKA activity in this context remain(s) elusive at the moment, non-canonical Wnt pathway appears to be one of the candidates, as Wnt signal has been shown to activate PKA signal for the patterning of the somite tissue,
and the distribution of phospho-CREB indicated high level of PKA activity both in the dorsal somite and dorsal neural tube of mouse embryos (Chen et al., 2005). Consistently, dorsal neural tube and premigratory neural crest cells express various Wnt genes (reviewed by Wu et al., 2003), although the shown Wnt pathway has been shown to promote EMT of trunk neural crest cells (Burstyn-Cohen et al., 2004).

We thank Drs. J. Weston and D. Newgreen for comments on the manuscript. We are grateful to Drs. Y. Nakaya, C.-M. Fan and R. Sekido, for technical suggestions and discussions. We thank Drs. C. Erickson, R. Lovell-Badge, H. Nakamura, P. Scotting, B. de Crombrugghe and K. Tamura for plasmids and antibodies. This work was supported in part by a grant to Y.W. from the Ministry of Education, Science, Sports and Culture, Japan (14034203, 14034205, 14017005, 13138201, 16015214, 16027201, 17024003).

References


