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

Daisuke Sakai[1], Takashi Suzuki[1], Noriko Osumi[1] and Yoshio Wakamatsu[2,*]

In neural crest formation, transcription factors, such as group E *Sox* and *Snail1*/*Snail2* (*Slug*) 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* is essential for BMP signal-mediated induction of *Snail2* and subsequent EMT in avian neural crest. We also show that Snail2 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, 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, S*ox9* 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 *PKIβ* were PCR-amplified from oligo (dT)-primed E2 embryo 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

[1]Center for Translational and Advanced Animal Research on Human Diseases, Division of Developmental Neuroscience, Graduate School of Medicine, Tohoku University, Sendai, Miyagi 980-8575, Japan. [2]Department of Developmental Neurobiology, Graduate School of Medicine, Tohoku University, Sendai, Miyagi 980-8575, Japan.

*Author for correspondence (e-mail: wakasama@mail.tains.tohoku.ac.jp)

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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* repression 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* D0.3 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*^{m1m2} were generous gifts from Dr B. de Crombrugghe (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 ^{32}P-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 poly (d(I-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, AACCCCCCTGCACCTGACTTGCG; mE-box2, AACCCCCCTGACCGAGACTTGCG; mE-box2(1), GAGCAGAAAACCCAACCCCCCTG; mE-box2(2), CACCTGACTTGCGGGTATTTACG; mE-box2(3), ACAACCCCTGCACCTGACTTGCG; mE-box2(4), AACCAAACTGCACCTGACTTGCG; mE-box2(5), AACCCCCAGTCACCTGACTTGCG.

Co-immunoprecipitation assay

Sox9 full coding sequence, N-terminal deletion (*Sox9ΔN*, carrying amino acids 253-494) and C-terminal deletion (*Sox9ΔC*, carrying amino acids 1-253) were subcloned into pyDF-HA for N-terminal HA-tagging (Fig. 5A). These constructs were transfected into COS7 cells with LipofectAMINE Plus reagent (Invitrogen). *Snail2* full coding sequence, N-terminal deletion (*Snail2ΔN*, corresponding to amino acids 121-268), and C-terminal deletion (*Snail2ΔC*, corresponding to amino acids 1-121) 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: GGAAAGCGACGAGGACAAA) as well as a mutated control dsRNA (GGATAGCCACGTGCAGAAA) were generated (Takara) and the dsRNAs (10 pM/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, CTCCTTCCTGGTCAAGAAAC; Snail2-R, CTTTCTGAACCGCTGTGATC; GAPDH-F, GTGAAAGTCGGAGTCAACGG; GAPDH-R, AGTTGGTGGTGCACGATGCA.

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 mm^2 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 (Morais 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

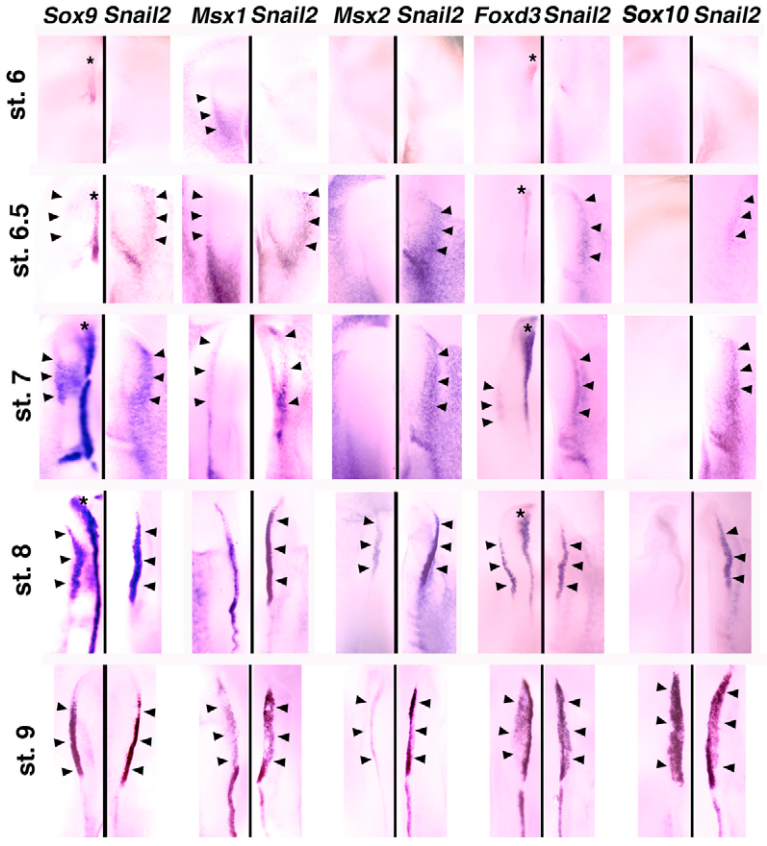

Fig. 1. Comparative analysis of the expression of transcription factors, such as *Sox9*, *Msx1*, *Msx2*, *Foxd3*, *Sox10* and *Snail2*, from a dorsal view. Stage 6-9 embryos were sagittally bisected, and the right halves were hybridized with *Snail2* probe, while the left halves were hybridized with 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

	HH stage				
	6	6.5	7	8	9
Snail2	–	+	+	++	+++
Sox9	–	+	+	++	+++
Msx1	+	+	+	++	++
Msx2	–	–	–	+	++
Foxd3	–	–	+	+	++
Sox10	–	–	–	–	+++

repressor form) was co-transfected with *GFP* into the ectoderm of cultured quail embryos (see Materials and methods) (see also Endo et al., 2002; Endo et al., 2003; Wakamatsu et al., 2004a; Sakai et al., 2005). Compared with the untransfected side of the neural fold, a clear reduction of *Snail2* mRNA expression was observed (5/6 cases, Fig. 2D). Such downregulation of *Snail2* was never observed when *GFP* was transfected alone (6/6 cases, Fig. 2D). As other group E *Sox* genes, such as *Sox8* and *Sox10*, were expressed in neural crest cells at later stages (Fig. 1) (see also Cheng et al., 2000; Cheung and Briscoe, 2003), transfected *Sox9-En-nuc* might have interfered with the function of *Sox8* and *Sox10* as well. In order to repress the expression of Sox9 specifically, we performed RNAi-mediated knockdown of *Sox9* by transfecting double-strand RNA (dsRNA, see Materials and methods), which was specific for *Sox9*, into neural plate explants cultured in the presence of BMP4. The transfection of *Sox9* dsRNA inhibited both Snail2 induction and EMT, otherwise promoted by BMP4 (Fig. 2E). The specificity of the effect of *Sox9* dsRNA was confirmed by the fact that the transfection of mutated dsRNA did not interfere with the promotion of Snail2 expression and EMT by BMP4, and that co-transfection of the *Sox9* dsRNA and the *Sox9* expression vector could cancel the effect of the *Sox9* dsRNA (Fig. 2E). *Sox10* co-transfection failed to cancel the effect of *Sox9* dsRNA, further showing the specificity of the *Sox9* RNAi (Fig. 2E). These results indicated the requirement of *Sox9* activity in *Snail2* expression and EMT. We thus conclude that *Sox9* is likely to contribute to the expression of *Snail2* and subsequent EMT in the neural crest cells by mediating BMP signal.

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. 3A), 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 (Msx1, 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

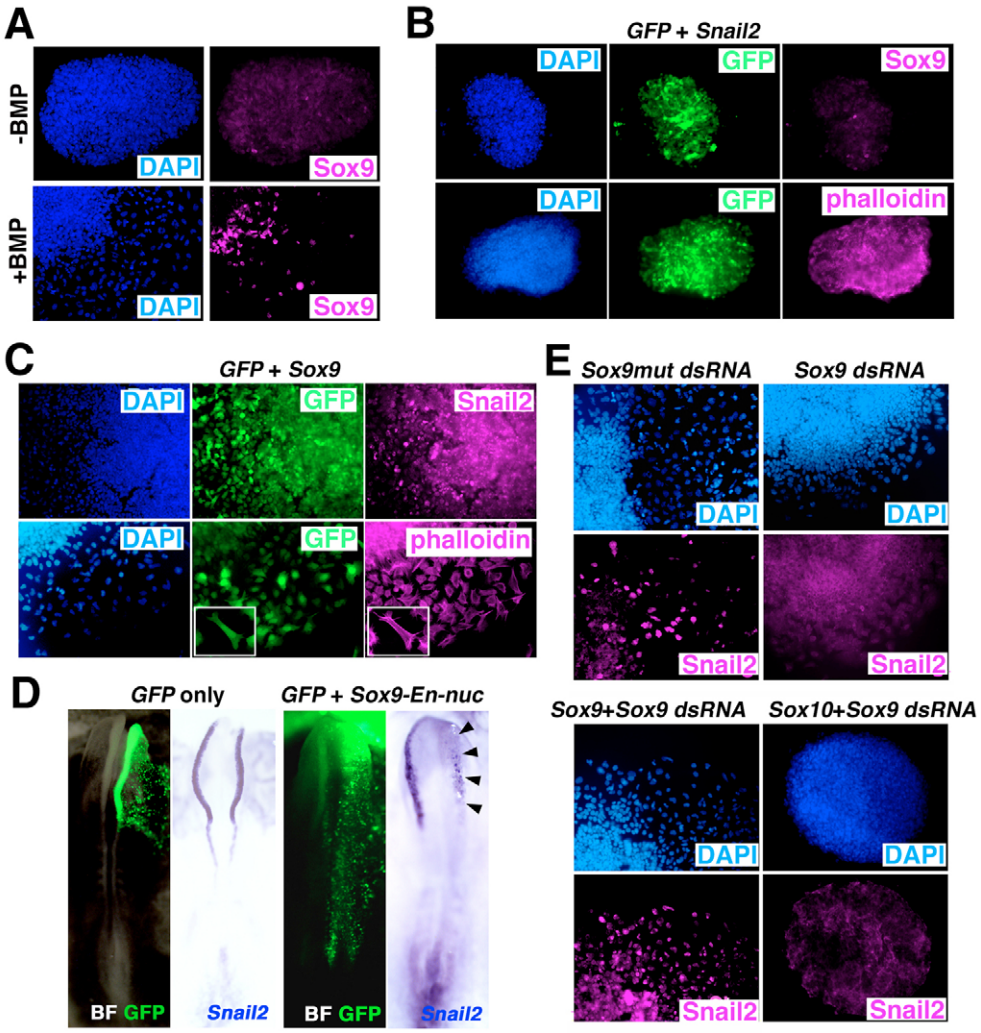

Fig. 2. *Sox9* is required for *Snail2* induction downstream of BMP signal. (**A**) BMP4 treatment (lower panels) of the neural plate explants effectively induces Sox9 expression and cell dispersal. (**B**) Neural plate explants were co-transfected with *Snail2* and *GFP* expression vectors, and stained 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 *Sox9-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.

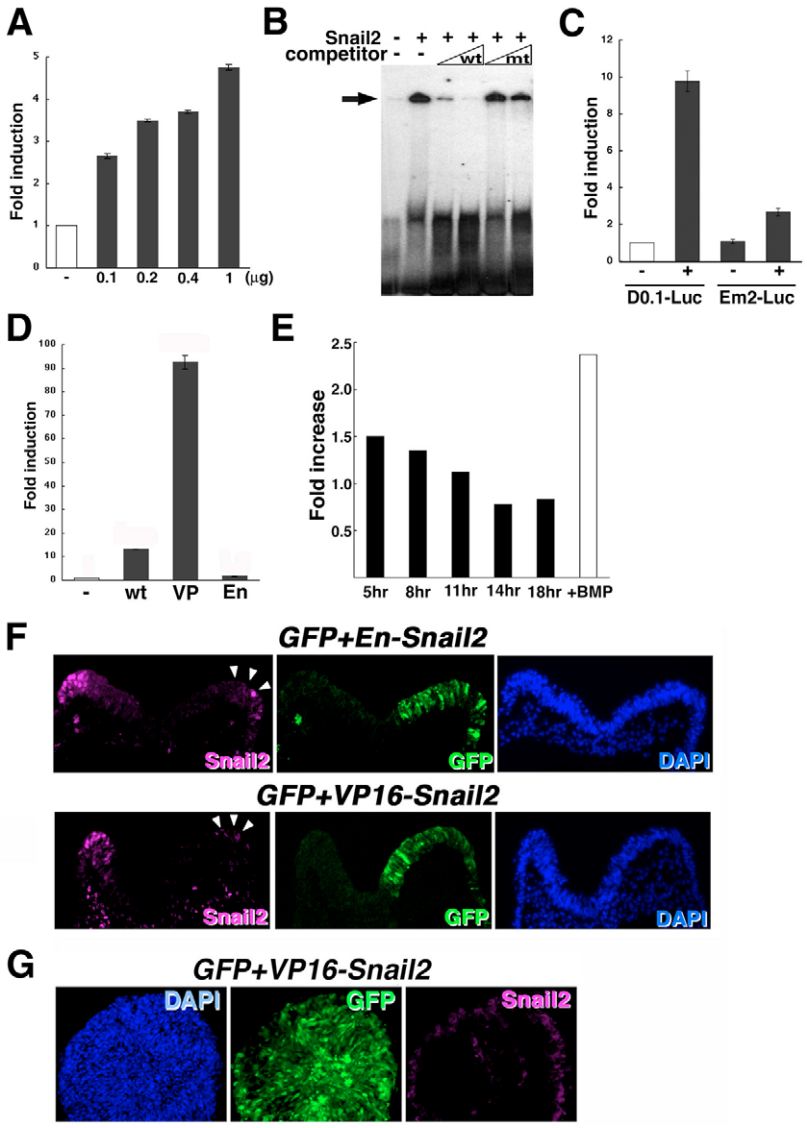

Fig. 3. *Snail2* activates its own promoter as a transcriptional activator. (**A**) A dose-dependent activation of D0.1-*Luciferase* reporter gene by *Snail2* in NIH3T3 cells. Different amounts of *Snail2* expression vector were co-transfected with the *Snail2* promoter-*Luciferase*. (**B**) Snail2 protein 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 activation 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.

activation of collagen 2a1 (*Col2a1*) enhancer/promoter in cartilage cells (Huang et al., 2000), and PKA activity appeared to be high in the dorsal neural tube of mouse embryos (Chen et al., 2005). To test the possible role of cAMP/PKA signal on *Sox9* function in neural crest development, we first treated neural plate explants either cultured in the presence of BMP4 or transfected with *Sox9*, with a PKA inhibitor H89 (Chijiwa et al., 1990), which reportedly inhibited emigration of crest cells from neural tube explants (Minichiello et al., 1999). Western blot analysis with anti-phospho-Sox9 antibody revealed that Sox9 phosphorylation by PKA was inhibited by H89 in 3T3 cells as expected (data not shown). H89 effectively inhibited the promotion of EMT by BMP4 and *Sox9* in a dose-dependent manner (Fig. 6A,B). However, a high dose of H89 might have inhibited other kinases non-specifically, we also took a different approach. Thus, a mutant version of *Sox9*, in which two PKA-mediated phosphorylation sites were mutated ($Sox9^{m1m2}$) (Huang et al., 2000), was transfected into the explants. This construct failed to induce EMT (Fig. 6C), suggesting the PKA-phosphorylation was required in this context. Interestingly, although explants cultured in the presence of BMP4 and H89 failed to express Snail2 (Fig. 6A), explants transfected with $Sox9^{m1m2}$, still expressed Snail2 (Fig. 6C). Thus, although the PKA activity is required for the induction of EMT by BMP4/Sox9, it seemed not to be essential for the *Snail2* induction by *Sox9*, 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 of cultured quail embryos. This forced activation of PKA signal induced ectopic expression of *Snail2* in the non-neural ectoderm and in the neural plate tissues (Fig. 8A; 6/8). As an opposite experiment, to interfere the PKA function in the neural fold, we transfected an expression vector of *PKIβ*, which encodes a potent PKA inhibitor protein (Van Patten et al., 1992; Scarpetta et al., 1993). The expression of *Snail2* was clearly decreased (Fig. 8B; 7/7), compared with the untransfected, contralateral neural folds, as well as the neural folds transfected

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 Brisco, 2003; McKeown et al., 2005).

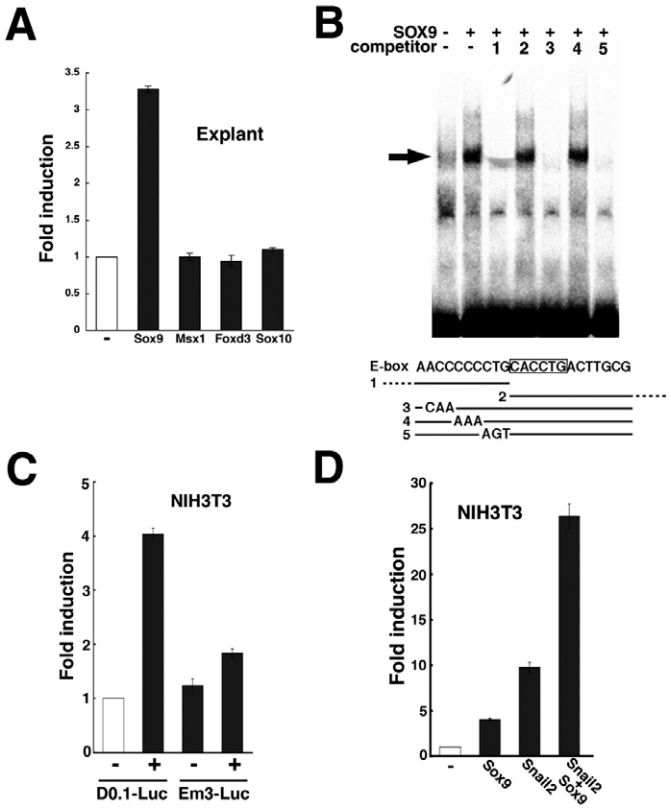

Fig. 4. *Sox9* directly activates *Snail2* promoter. (**A**) Activation of *Snail2* promoter by *Sox9* in neural plate explants. D1.2-*Luciferase* reporter gene and other 'crest-specific' transcription factors were electroporated into neural plates, and cultured for 20 hours without BMP4. Only *Sox9* can activate the reporter. Results are shown as fold-induction compared with the result obtained from explants, transfected with D1.2-*Luciferase* and empty pyDF30 plasmid. (**B**) Sox9 binds to the flanking sequence of E-box2. EMSA was performed to determine the binding site of Sox9 on *Snail2* promoter. The sequence and the position of the probe and competitors are shown below. E-box2 is indicated by an open box. The Sox9 binding to the E-box probe is interfered by the addition of competitor 1, 3 and 5, but not by competitor 2 or 4. (**C**) The flanking sequence of E-box2 is required for the *Snail2* promoter activation by Sox9. When the C-rich sequence next to the E-box2 in the D0.1 reporter is mutated (Em3), activation level of the promoter by Sox9 (+) is significantly decreased, compared with the wild-type D0.1. (**D**) *Sox9* and *Snail2* synergistically activate D0.1-*Luciferase* promoter. D0.1-*Luciferase* was co-transfected with *Sox9* or *Snail2* (or both) into NIH3T3 cells. Results are shown as a fold-induction compared with the result obtained from cells transfected with D0.1-*Luciferase* and an empty pyDF30.

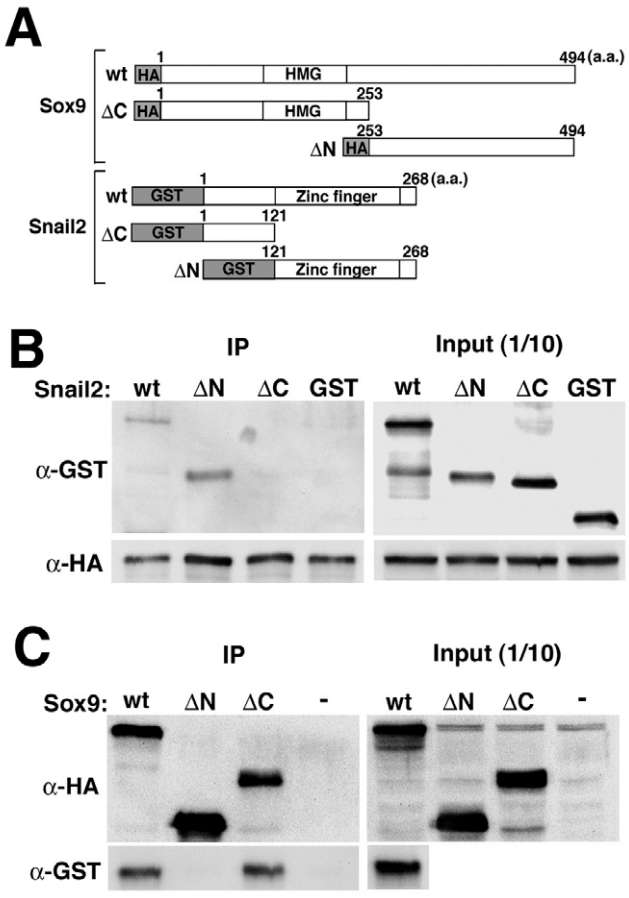

Fig. 5. Sox9 and Snail2 form a protein complex. (**A**) Schematic diagram of wild type (wt) and deletion mutants (ΔC and ΔN) of HA-tagged Sox9 and GST-tagged Snail2. (**B**) Interaction of GST-Snail2 deletions and full-length HA-Sox9. Extract of HA-Sox9-transfected COS7 cells were incubated with full-length (wt) and deletions of GST-Snail2 (ΔN and ΔC), and immunoprecipitated with anti-HA beads. Full-length and ΔN-Snail2 is detected in precipitated fractions by anti-GST. (**C**) Interaction of full-length GST-Snail2 and deletions of HA-Sox9. Extracts of COS7 cells transfected with HA-tagged full-length (wt) Sox9 or deletions (ΔN and ΔC) were incubated with GST-Snail2 (ΔN) and immunoprecipitated with anti-HA beads. With anti-GST, precipitation of Snail2 is detected when incubated with extracts containing either full-length Sox9 (wt) or ΔC-Sox9.

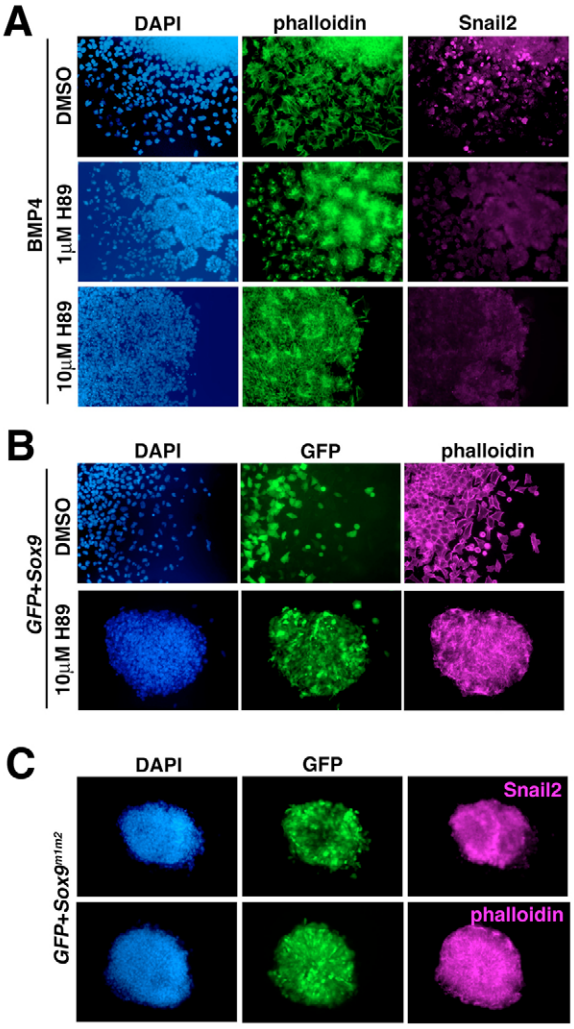

Fig. 6. PKA signal is required for BMP4 and Sox9 to induce neural crest cells in neural plate explants. (**A**) Effects of H89, a PKA inhibitor, on BMP4-mediated induction of neural crest. Neural plate explants were cultured in the presence of BMP4, and F-actin (phalloidin) and Snail2 were examined. Whereas explants treated with a vehicle (DMSO) show extensive EMT and Snail2 expression, treatment with H89 at low (1 μM) and high (10 μM) concentrations produces partial and nearly complete inhibition of EMT, respectively. Under both conditions, Snail2 induction by BMP4 is blocked. (**B**) H89 inhibits Sox9-mediated induction of EMT. Although *Sox9* transfection strongly induces EMT in neural plate explants, indicated by an extensive cell dispersal in the presence of DMSO, H89 completely blocked EMT. (**C**) Transfection of $Sox9^{m1m2}$, a PKA-mediated phosphorylation-deficient mutant, induces Snail2 expression, but fails to promote EMT.

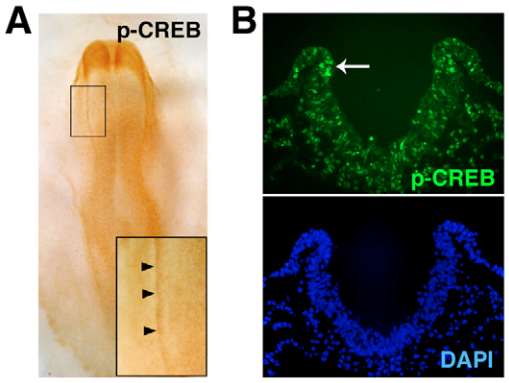

Fig. 7. Tissue distribution of phospho-CREB in stage 7 embryos. (**A**) A whole-mount preparation of anti-phospho-CREB stained embryo. Although anti-phospho-CREB immunoreactivity is broadly observed, stronger and weaker staining can be seen in the neural folds and midline tissue, respectively. The inset indicates a magnified view of boxed area and the neural fold is indicated by arrowheads. (**B**) Anti-phospho-CREB staining on section. Higher levels of CREB phosphorylation are observed in the prospective neural crest cells in the neural fold (arrow). DAPI staining shows cell nuclei.

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 groupE 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 groupB1 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

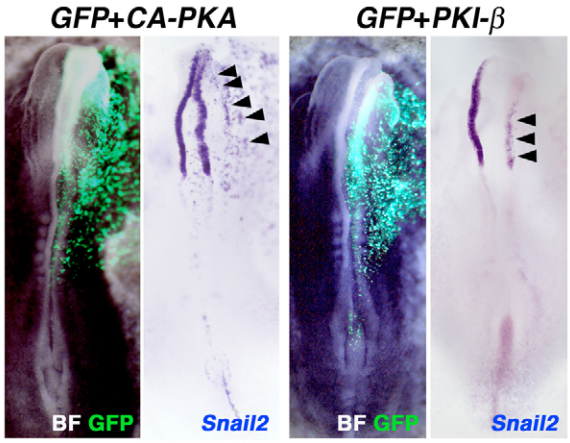

Fig. 8. PKA signal is important for *Snail2* expression in the neural crest. Transfection of *CA-PKA* in the ectoderm of cultured embryo induces ectopic *Snail2* expression in neural plate and non-neural ectoderm areas (arrowheads). By contrast, misexpression of *PKIβ* expression vector reduces the *Snail2* mRNA expression, compared with the untransfected side. Electroporation of *GFP* and *CA-PKA* or *PKIβ* expression constructs into the ectoderm was performed on quail embryos at stage 5, and the embryos were cultured for 7 hours.

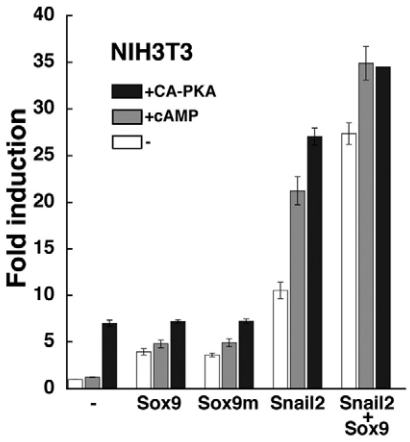

Fig. 9. Enhancement of Snail2-mediated activation of the *Snail2* promoter by PKA signal. *Sox9*, *Sox9^{m1m2}*, *Snail2* or *Sox9+Snail2* were transfected into NIH3T3 cells along with D0.1-*Luciferase* reporter. To activate PKA signal, either 1 mM of 8-bromo-cAMP (+cAMP) was added in culture or constitutively active PKA (CA-PKA) was co-transfected. Results are shown as a fold-induction compared with the result obtained from cells transfected with D0.1-*Luciferase* and an empty pyDF30 vector.

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 phspho-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,

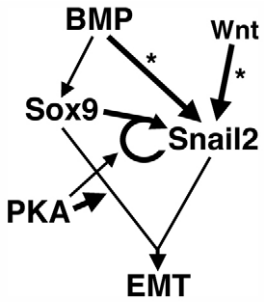

Fig. 10. Regulatory relationship of signals and transcription factors in avian cranial neural crest formation and EMT. Thick arrows indicate direct activation. Asterisks indicate direct activation of *Snail2* transcription reported in our previous paper (Sakai et al., 2005).

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 canonical Wnt pathway has been shown to promote EMT of trunk neural crest cells (Burstyn-Cohen et al., 2004).

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