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ARTICLE

Multiple Roles of SOX2, an HMG-Box Transcription Factor in Avian Neural Crest Development

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Expression of *Sox2*, which encodes an HMG-box–type transcription factor, is down-regulated in the neural plate when neural crest segregates from dorsal neural tube and remains low during crest cell migration. *Sox2* expression is subsequently up-regulated in some crest-derived cells in the developing peripheral nervous system and is later restricted to glial sublineages. Misexpression of *Sox2* and mutant forms of *Sox2* both in neural plate explants and in embryonic ectoderm reveals that *Sox2* inhibits neural crest formation as a transcriptional activator. Similar manipulation of *Sox2* function in migratory and postmigratory neural crest-derived cells indicates that *Sox2* regulates proliferation and differentiation in developing peripheral nervous system. *Developmental Dynamics 229:74–86, 2004.* © 2003 Wiley-Liss, Inc.

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INTRODUCTION

Neural crest cells give rise to a variety of cell types, including neurons and glia in the peripheral nervous system (PNS), and connective tissues of the craniofacial structures. Neural crest is initially formed at the junction of epidermal and neural ectoderm by the mutual interaction of these tissues (Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996). Subsequently, neural crest cells undergo epithelial–mesenchymal transition (EMT), and migrate extensively in the embryonic environment. Neural crest cells can be identified in the neural fold of avian and *Xenopus* embryos by the expression of a Zn-finger transcription factor, *Slug* (Nieto et al., 1994; Linker et al., 2000), before EMT. *Slug* has been shown to regulate neural crest formation and subsequent EMT (Nieto et al., 1994; LaBonne and Bronner-Fraser, 2000; del Barrio and Nieto, 2002).

In the trunk of avian and mammalian embryos, crest-derived neuroglial progenitors migrate medially and give rise to dorsal root ganglia, sympathetic ganglia, and Schwann cells along the spinal nerve. These peripheral ganglia include both glial cells and neurons. Regulatory mechanisms of fate determination and differentiation of crest-derived progenitors have been studied extensively. Both environmental cues and crest cell-intrinsic factors appear to affect these processes (see review; Anderson, 1997). For glial development, NEUREGULIN-1 has been shown to promote glial differentiation, proliferation, and survival (Shah et al., 1994; Dong et al., 1995). However, such environmental factors do not appear to be sufficient in ganglionic locations, where both neurons and satellite glia differentiate. In a previous study, we showed that NOTCH-mediated lateral inhibition is involved in this process (Wakamatsu et al., 2000). *Notch1*, which encodes a membrane-bound receptor, is expressed in undifferentiated crest-de-

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rived cells of the developing ganglia. A membrane-bound NOTCH ligand, DELTA1, is expressed in early neuronal cells. Continuous activation of NOTCH signaling by misexpression of the intracellular domain of NOTCH1 inhibited neuronal differentiation of cultured crest cells, suggesting that neuron-derived DELTA1 activates NOTCH1 of neighboring cells, and thereby inhibits their neuronal differentiation. Furthermore, it has been shown that a transient activation of NOTCH signaling of neural crest-derived cells with DELTA1-Fc fusion protein promotes glial differentiation (Morrison et al., 2000).

SOX family transcription factors possess an HMG type DNA-binding motif, and more than 30 of them have been identified in vertebrates (Pevny and Lovell-Badge, 1997; Schepers et al., 2002). Although the spatiotemporal expression of *Sox* genes has not been fully documented, some of these genes are expressed in neural crest and its derivatives. The best-described example is *Sox10*, a member of subgroup E of *Sox* genes. *Sox10* is expressed in early migrating crest cells and its expression is subsequently restricted to glial cells (Bondurand et al., 1998; Cheng et al., 2000). Mice carrying a mutation in *Sox10*, *dominant megacolon*, have defects in melanocytes and the enteric nervous system (Southard-Smith et al., 1998). Similar defects were observed in *Sox10* mutant zebrafish, *colorless* (Kelsh and Eisen, 2000; Dutton et al., 2001). More recently, targeted *Sox10* mutant mice, as well as the *dominant megacolon* mutant have been shown to have severely reduced PNS (Britsh et al., 2001). Detailed analyses in cultured crest-derived cells indicated that *Sox10* is required for both survival of crest-derived cells and their glial differentiation (Paratore et al., 2001) and is required for a maintenance of multipotency of autonomic progenitors (Kim et al., 2003). *Sox2*, a member of subgroup B, is expressed in the neural epithelium of the chick central nervous system (Uwanogho et al., 1995; Uchikawa et al., 1999), and is involved in neural differentiation of *Xenopus* (Mizuseki et al., 1998; Kishi et al., 2000). In a previous study, we briefly reported that *Sox2* is expressed in the PNS cells (Wakamatsu et al., 2000). The property of SOX2 as a transcriptional activator has been well studied in regulation of *δ1-crystallin* and *FGF4* expressions (see review, Kamachi et al., 2000; FGF, fibroblast growth factor). In both cases, SOX2 activates these genes in association with other transcription factors, such as PAX6 for a *δ1-crystallin* enhancer (Kamachi et al., 1995, 1998, 2001), and OCT3/4 for a *FGF4* enhancer (Yuan et al., 1995). Such interaction of SOX proteins with other transcription factors may be a common feature of this family. For example, SOX10 has also been shown to coactivate the *mitf* promoter with PAX3 (Verastegui et al., 2000).

In this study, we report (1) that *Sox2* inhibits neural crest formation and subsequent EMT; and (2) that, in later stages of development, *Sox2* inhibits neuronal differentiation downstream of NOTCH signaling.

RESULTS

Expression of *Sox2* During Neural Crest Development

To elucidate the function of *Sox2* in neural crest development, we first examined *Sox2* mRNA and protein distribution in avian embryos from stage 6 to stage 30 (Hamburger and Hamilton, 1951). First, expression patterns of *Sox2* and a neural crest marker *Slug* (Nieto et al., 1994) were compared on neighboring sections of zero-, two-, and six-somite stage embryos (stage 6–8^{+}) by in situ hybridization (Fig. 1). At the zero-somite stage, *Sox2* expression was observed in the prospective neural plate, but the boundary of *Sox2*-positive area and -negative area (prospective epidermis) was not sharp (Fig. 1A). At this stage, almost no expression of *Slug* was detectable in the ectoderm, while some expression was seen in the mesodermal mesenchyme (Fig. 1B). At the two-somite stage, *Slug* expression became evident in the head neural fold, indicating a generation of premigratory neural crest (Fig. 1D). At this stage, *Sox2* expression was higher in the medial neural plate, and lower expression of *Sox2* was also detectable in the region that expressed *Slug* (Fig. 1C). It seems reasonable that *Slug* is only expressed at regions of low *Sox2* expression. At the six-somite stage, as left and right head neural folds came in contact, *Sox2* expression diminished in the *Slug*-expressing crest cells (Fig. 1E,F). These observations suggest that *Sox2* expression is down-regulated when the neural plate/neural tube cells acquire the neural crest fate.

Similar to the head region, at stage 13, SOX2 protein expression was low in the SLUG-expressing premigratory neural crest cells within the trunk neural tube, compared with SOX2 expression in SLUG-negative neural tube cells (Fig. 2A,B). In stage 14–17 embryos, SOX2 protein expression could not be detected in migrating crest-derived cells, while SOX2 expression was clearly observed in the neural tube (Fig. 2C). As previously reported at the mRNA level (Wakamatsu et al., 2000), SOX2 expression become evident in nascent dorsal root ganglia (DRG) at stage 22–25 and was mostly restricted to the periphery of the DRG (Fig. 2D). Crest-derived cells in this peripheral region of the developing DRG actively proliferate and generate both neurons and glia (Wakamatsu et al., 2000). *Sox2* expression appeared to be down-regulated in neuronal cells in the core region of the DRG. Thus, SOX2 protein could not be detected in neuronal cells (Fig. 2D′) defined by expression of specific neuronal markers, such as immunoreactivities of neuron-specific type III β-TUBULIN (Lee et al., 1990) and Hu (Marusich et al., 1994; Wakamatsu and Weston, 1997; Wakamatsu et al., 2000; see also Fig. 2H).

At later stages of development, two crest-derived glial sublineages, satellite cells intermingled with neurons in ganglia, and Schwann cells along the spinal nerve expressed SOX2 protein (Fig. 2E,F,H). Schwann cells expressing a glial marker P0 (Bhattacharyya et al., 1991) coexpressed SOX2 (Fig. 2F) from stage 24, consistent with the previous observation of coexpression of SOX2 and P0 in cultured crest cells (Wakamatsu et al., 2000). From stage 18,

melanocyte precursors disperse on the lateral migration pathway between epidermis and dermomyotome (Kitamura et al., 1992; Wakamatsu et al., 1998), but no *Sox2* mRNA could be detected in cells on this pathway (data not shown). Consistently, melanocyte precursors expressing MMP115, did not possess anti-SOX2 immunoreactivity at the stages examined (stage 18–26; data not shown). We have also examined the ectomesenchyme of cranial region, and no *Sox2* expression was detected, as reported (Uchikawa et al., 1999). Taken together, *Sox2* expression appears to be up-regulated in the crest-derived cells, which contribute to the PNS, and subsequently to be restricted to glial sublineages.

Sox2 Inhibits Neural Crest Formation in the Neural Fold

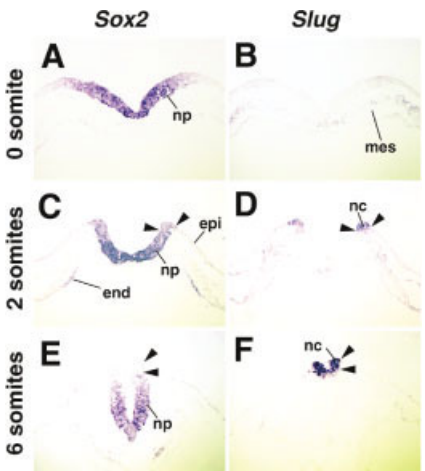
To study the function of *Sox2* in neural crest formation, an expression vector of FLAG-epitope–tagged full-length SOX2 was prepared (Fig. 3). Because *Sox2* expression was detected in the neural plate and neural tube but was clearly reduced in premigratory *Slug*-positive neural crest, we examined the effect of *Sox2* misexpression in the neural crest formation. Stage 5–6 quail embryos were transfected with FLAG-tagged *Sox2* expression vector (Fig. 3) along with *GFP* (green fluorescent protein) expression plasmid in the right side of the ectoderm, including the future neural fold (Endo et al., 2002; see also Experimental Procedures section). After 9 hr of embryo culture, neural crest formation was assessed by the expression of *Slug*. In contrast to the normal induction of *Slug* expression in the contralateral, untransfected neural fold or in the right neural fold of embryos transfected only with *GFP*, *Slug* expression was severely reduced when *Sox2* was transfected (Fig. 4A–D). To examine the effect in detail, *FLAG-Sox2*–transfected embryos, after 9 hr of culture, were sectioned and double-stained with anti-FLAG and anti-SLUG antibodies (Fig. 4E–H). Compared with the untransfected side (contralateral; Fig. 4E,F), the number of SLUG-positive cells were reduced, and most of the FLAG-SOX2–positive cells were SLUG-negative (ipsilateral; Fig. 4G,H). It is likely, therefore, that *Sox2* misexpression repressed SLUG expression cell autonomously. Con-

Fig. 1. Comparison of *Sox2* and *Slug* expression in neural crest formation in the head neural fold. **A–F:** Transverse neighboring sections of zero-, two-, and six-somite stages of quail embryos (corresponding to Hamburger and Hamilton stage 6, 7, and 8, respectively) were hybridized with *Sox2* (A,C,E) and *Slug* (B,D,F) probes. At all stages examined, *Sox2* expression was mainly observed in prospective neural plate (np). At the two-somite stage, *Sox2* expression overlaps with *Slug* expression at the head neural fold, suggesting that premigratory neural crest cells (nc, arrowheads) express both genes. At the six-somite stage, however, *Sox2* expression is down-regulated in *Slug*-positive crest cells. *Slug* expression is also observed in mesodermal mesenchyme (mes), and *Sox2* expression is detected in a subpopulation of endodermal cells (end). epi, epidermis

Fig. 2. *Sox2* expression in the trunk neural crest cells and in the developing peripheral nervous system. **A,B:** A transverse section of stage 13 (E2) embryo. Anti-SOX2 immunoreactivity is low in premigratory neural crest cells (A), which are identified with anti-SLUG staining in B (blue–green, arrowhead). Most of the neural tube (nt) cells possess SOX2 in their nuclei. The overlap of SLUG (fluorescein isothiocyanate in green) and DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride; blue) makes SLUG staining blue–green. **C:** A transverse section of stage 17 (E3) embryo. SOX2 protein is undetectable in migratory crest cells (nc), which are stained with HNK1 antibody. **D,E:** SOX2 protein expression in non-neuronal cells of developing peripheral nervous system at stage 23 (E4). Nuclear staining of anti-SOX2 (pink) is mostly detected in the periphery of the nascent dorsal root ganglia (drg, DRG; D). **D′:** A high magnification view of boxed area in D. DRG neurons (arrows) expressing neuron-specific type III β-TUBULIN (TuJ1, green) do not have SOX2 in their nuclei. The TuJ1-negative peripheral cells possess SOX2 (arrowheads). E: Schwann cells along the TuJ1-positive axons also possess SOX2 immunoreactivity. The overlap of SOX2 (cy3 in red) and DAPI (blue) makes SOX2 staining pink. **F:** P0-positive Schwann cells express SOX2. A spinal nerve of stage 28 (E6) embryo is shown. Most of nuclei within the P0-positive nerve cord (green) possess SOX2 immunoreactivity (arrowheads). The overlap of SOX2 (cy3 in red) and DAPI (blue) makes SOX2 staining pink. **G,H:** High magnification of stage 30 (E6.5) DRG, showing the same field. SOX2-positive nuclei (pink, arrowheads in H) localize between Hu-positive neurons (green cell bodies in H, arrows). Smaller nuclei of these SOX2-positive cells (arrowheads in G), along with their localization between neurons and with no expression of neuronal marker, suggest that these SOX2-positive cells are satellite glia.

Fig. 3. *Sox2* expression constructs.

Fig. 4. *Sox2*-misexpression in the head neural fold inhibits neural crest formation and epithelial–mesenchymal transition (EMT). **A–D:** Electroporation of *GFP* (A,B) or *GFP* + *FLAG-Sox2* (C,D) expression vectors was performed on stage 5 embryos, and the embryos were cultured for 9 hr until stage 9. A,C: Transfected areas are visualized by the fluorescence of green fluorescent protein (GFP) overlaid on brightfield image of electroporated live embryos. B,D: *Slug* mRNA expression in the head fold are shown at higher magnification, corresponding to the boxed areas of A and C, respectively. Misexpression of *Sox2* reduced the expression of *Slug* (arrowheads in D). **E,F:** *Sox2*-transfected ectoderm cells fail to express SLUG protein. *Sox2*-transfected embryos, similar to C and D, were cross-sectioned and stained with anti-FLAG (green), anti-SLUG (red), and DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride; blue). In the contralateral (untransfected) neural fold, many SLUG-positive premigratory neural crest nuclei are observed. **G:** In contrast, on the ipsilateral side, the number of SLUG-positive nuclei is reduced. **H:** Many FLAG-positive nuclei of *Sox2*-transfected cells can be identified, and most of the FLAG-positive nuclei do not possess SLUG immunoreactivity (H, arrowheads). **I,J:** *Sox2*-transfected cells fail to undergo EMT from the ectoderm. Electroporation of *GFP* + *FLAG-Sox2* expression vectors was performed on stage 5 embryos, and the embryos were cultured for 24 hr. I indicates the morphology of the cross-section with differential interference contrast (DIC) optics. J is the same section of I and shows FLAG-positive, *Sox2*-transfected cells remaining dorsally (green), and HNK-1–positive, untransfected crest cells colonized in the branchial arch primordia (red, arrowheads). **K:** A higher magnification of boxed area in J. **L:** A neighboring section of H, showing that some *Sox2*-transfected cells (green, arrowheads) successfully escaped from the ectoderm but failed to express HNK-1. Arrows indicate HNK-1–positive crest cells migrating underneath the epidermis. np, neural plate.



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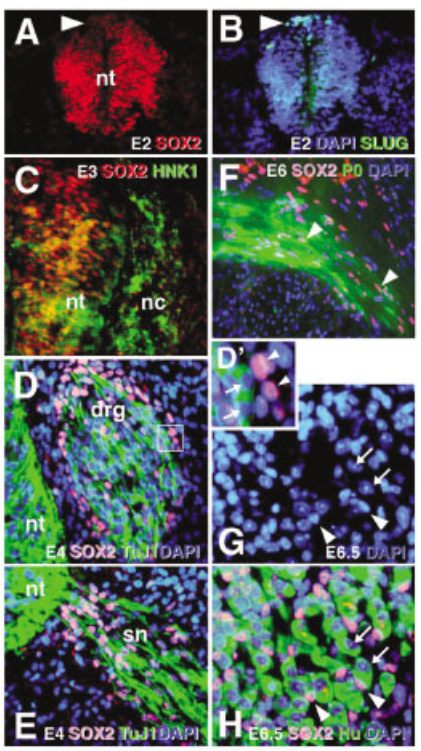

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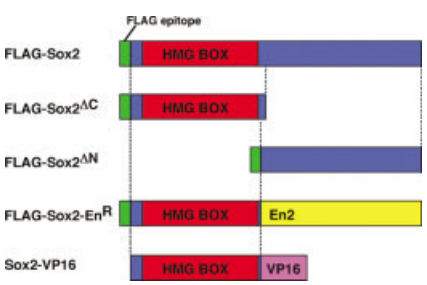

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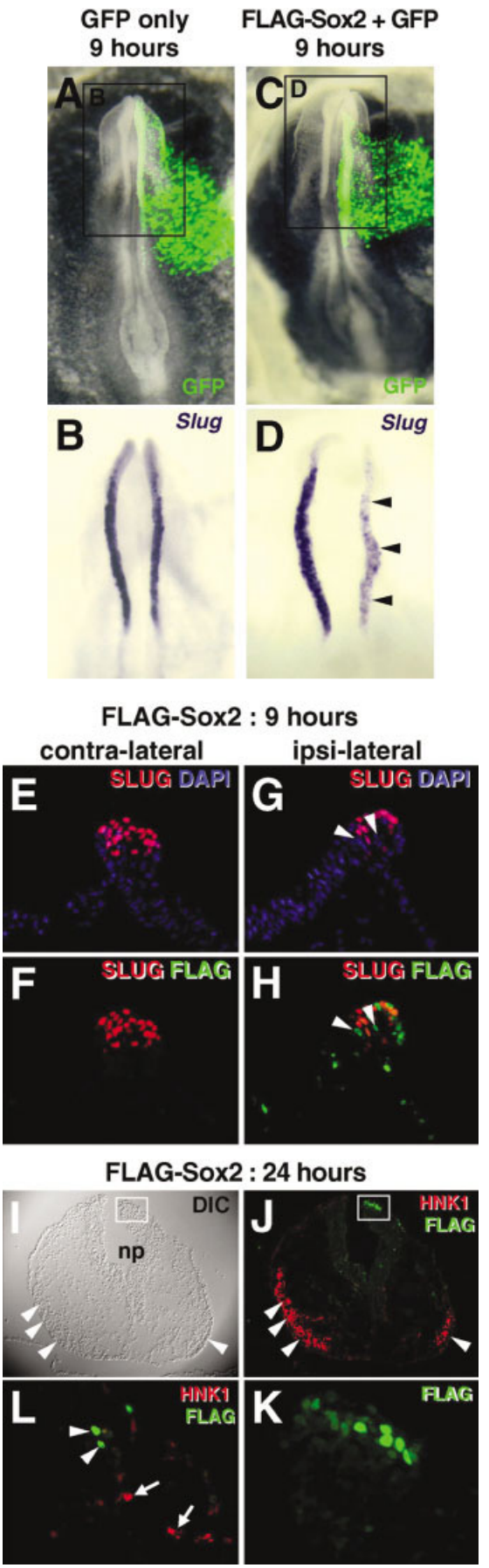

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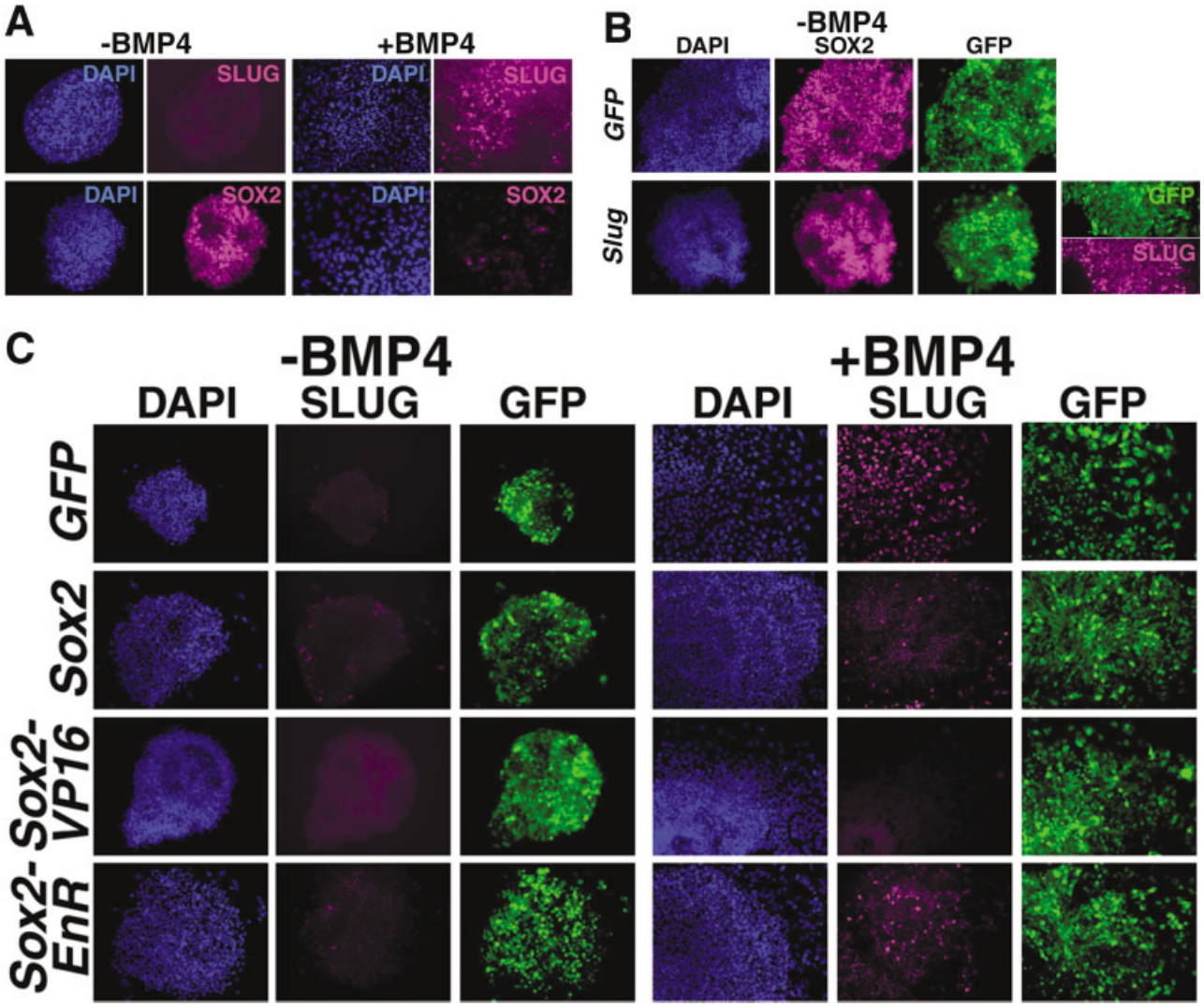

Fig. 5.

sistent with the decreased *Slug* expression, 24 hr after electroporation, most of the *Sox2*-transfected cells in the head neural fold failed to undergo EMT (Fig. 4I–K). Consequently, the number of HNK-1–positive migrating crest cells was severely decreased on the transfected side of the branchial arch primordia, compared with untransfected side (Fig. 4J). Although there were a few *Sox2*-transfected cells successfully emigrated, they did not express HNK-1 and failed to enter the arch primordia (Fig. 4L). These results suggest that *Sox2* prevents neural crest formation and subsequent migration.

To further understand the involvement of *Sox2* in neural crest formation, we used neural plate explant culture for a variety of assays. Intermediate neural plates were excised from stage 6–7 quail embryos at the

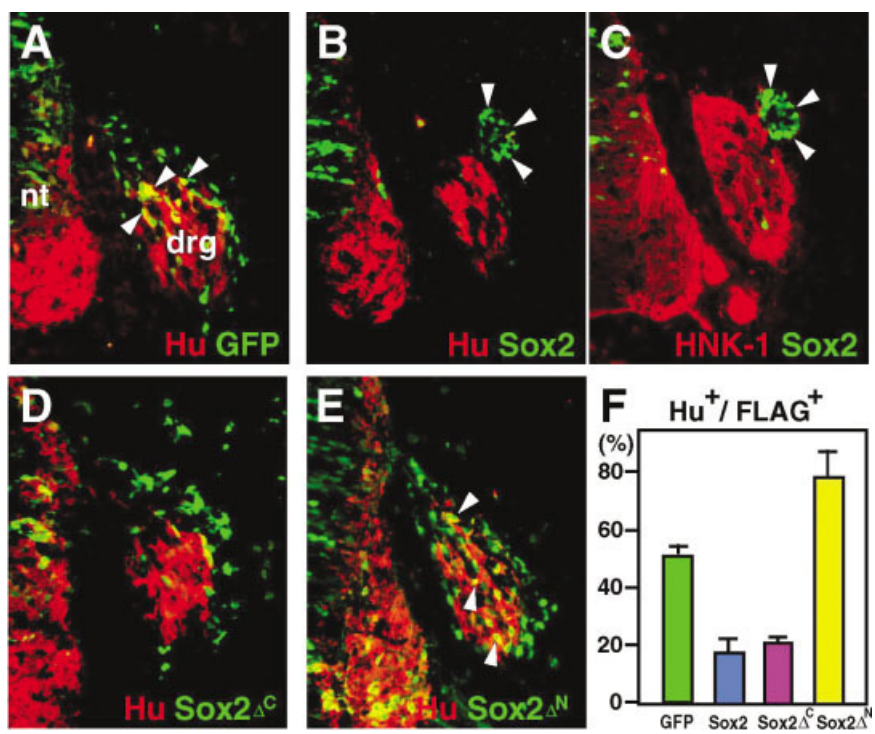

Fig. 6.

prospective midbrain level (see Experimental Procedures section). As previously shown (Liem et al., 1994; Selleck and Bronner-Fraser, 1995), while in the absence of bone morphogenetic protein-4 (BMP4), the neural plate stayed as aggregates and no SLUG immunoreactivity was observed; by adding BMP4 and N2 supplement in culture media, the explants were flattened, cells dispersed, and many of them expressed SLUG (Fig. 5A). In this condition, strong SOX2 immunoreactivity was detected without BMP4, and the addition of BMP4 clearly down-regulated SOX2 (Fig. 5A). To test whether *Slug* induced by BMP4 repressed *Sox2* expression, a *Slug* expression vector was transfected by electroporation into the neural plate explant (see Experimental Procedures section). Transfection of *GFP* alone or *GFP* + *Slug* had no effect on SOX2 expression (Fig. 5B). Thus, BMP signaling causes the down-regulation of *Sox2* in neural crest territory, independent of *Slug* activity.

As *Sox2* misexpression in the embryonic ectoderm inhibited *Slug* induction, we tested *Sox2* misexpression in neural plate explants (Fig. 5C). *Sox2* misexpression interfered with the induction of SLUG by BMP4 (Fig. 5C). Because *Sox2* fused with transcription activation domain of VP16 (Kamachi et al., 1999) also inhibited SLUG expression (Fig. 5C), as well as subsequent EMT (Fig. 5C), *Sox2* likely acted as a transcriptional activator to repress *Slug*. Consistently, *Sox2* fused to *Engrailed* repressor domain (*Sox2En*^{R}) did not interfere the SLUG induction by BMP4. It was also noted that a manipulation of *Sox2* function was not sufficient for SLUG activation, as none of the *Sox2* constructs promoted SLUG expression in the absence of BMP4 (Fig. 5C).

Sox2 Regulates Neural Crest Differentiation In Vivo

To study the function of *Sox2* in PNS development, we first performed in ovo electroporation into chick trunk neural tube, before the neural crest migration (Funahashi et al., 1999). The FLAG-tagged *Sox2* expression constructs were co-injected with *GFP* plasmid into the lumen of the neural tube of stage 13–14 chicken embryos at the level of last five somites after which, electroporation was performed (see Experimental Procedures section). We also prepared additional *Sox2* mutant constructs, such as the N-terminal half of SOX2, including HMG-box (*Sox2*$^{\Delta C}$), and the C-terminal half of SOX2 without HMG-box (*Sox2*$^{\Delta N}$).

Forty-eight hours after the electroporation into the trunk neural tube, neuronal differentiation of transfected cells were examined in histologic sections (Fig. 6). At this stage (stage 22–23), crest-derived cells, which have already taken a neuronal fate, express Hu immunoreactivity and form a neuronal core in the center of the developing DRG, while undifferentiated crest-derived cells surround the core (Wakamatsu and Weston, 1997; Wakamatsu et al., 2000). In *GFP*-transfected embryos, 51% of transfected cells in the DRG expressed Hu (Fig. 6A,F). Although only a small fraction of *Sox2*-transfected cells could migrate because of the inhibitory effect of *Sox2* on migration (see above), transfected cells, which successfully escaped from the neural tube, were found often as aggregates in the medial pathway (Fig. 6B,C). The *Sox2*-transfected cells in the aggregate weakly expressed HNK-1 (Fig. 6C), and only a few of them expressed Hu (18%; Fig. 6B,F). Although many *Sox2*$^{\Delta C}$-transfected cells remained in the dorsal neural tube, some of them colonized the DRG (Fig. 6D). Expression of *Sox2*$^{\Delta C}$ inhibited neuronal differentiation (23%; Fig. 6D,F), but, unlike full-length *Sox2*-transfected cells, *Sox2*$^{\Delta C}$-transfected cells did not form aggregates (Fig. 6D). In contrast to *Sox2* and *Sox2*$^{\Delta C}$, many *Sox2*$^{\Delta N}$-transfected cells were found in DRG and showed an increase of neuronal differentiation (78%; Fig. 6E,F).

Because misexpression of *Sox2-VP16* severely interfered with EMT, and because misexpression of *Sox2-En*^{R} caused extensive cell death immediately after the EMT, we could not assay the effect of these constructs on the differentiation of transfected crest-derived cells (data not shown). Taken together, nevertheless, *Sox2* seems to regulate the ability of crest-derived PNS cells to coalesce into DRG and to differentiate.

Fig. 5. Regulatory relationship of bone morphogenetic protein (BMP) signal, *Slug*, and *Sox2*. **A:** Intermediate neural plates of stage 6–7 quail embryos were cultured with or without BMP4 (and N2 supplement) and immunostained with anti-SLUG and SOX2 antibodies, followed by DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride) nuclear staining. In the absence of BMP4, neural plate tissues remain as aggregates and express SOX2 protein, but no SLUG expression is detected. In the presence of BMP4, explant cells dispersed, SLUG expression is induced, and SOX2 is down-regulated. **B:** Neural plates were transfected either with *GFP* expression vector alone or with both *GFP* and *Slug* expression vectors, and SOX2 expression was examined. *Slug* misexpression fails to down-regulate SOX2 expression in the absence of BMP4. Transgene-derived SLUG protein expression was confirmed by immunostaining of transfected explants with anti-SLUG antibody. **C:** Neural plates were transfected with *Sox2* expression vectors along with *GFP* plasmid, and SLUG expression was examined. In the absence of BMP4, none of *Sox2* constructs can induce SLUG expression or cell dispersal. Full-length *Sox2* and *Sox2-VP16* fusion inhibit the induction of SLUG by BMP4, while *Sox2-En*^{R} fusion has little effect. GFP, green fluorescent protein.

Fig. 6. Misexpression of *Sox2* constructs affects neuronal differentiation in vivo. Neural tubes of E2 embryos were electroporated and fixed 48 hr later. **A:** *GFP*-transfected crest-derived cells (green) colonized nascent dorsal root ganglia (drg), and some of them express a neuronal marker, Hu (red). Overlap of green and red appears yellow (arrowheads). nt, neural tube. **B,C:** *Sox2*-transfected cells (FLAG in green) form aggregates nearby but not within the ganglia (arrowheads). Most of transfected cells are Hu-negative. **D:** Most of *Sox2*$^{\Delta C}$-transfected cells (FLAG in green) are Hu-negative. **E:** Many *Sox2*$^{\Delta N}$-transfected cells (FLAG in green) express Hu (arrowheads, yellow). **F:** Proportion of Hu-positive neuronal cells among transfected cells in the drg. Three embryos were examined for each condition, and the average is shown. To obtain each value, more than 200 FLAG-positive cells were examined in each embryo. Error bars indicate standard deviation. GFP, green fluorescent protein.

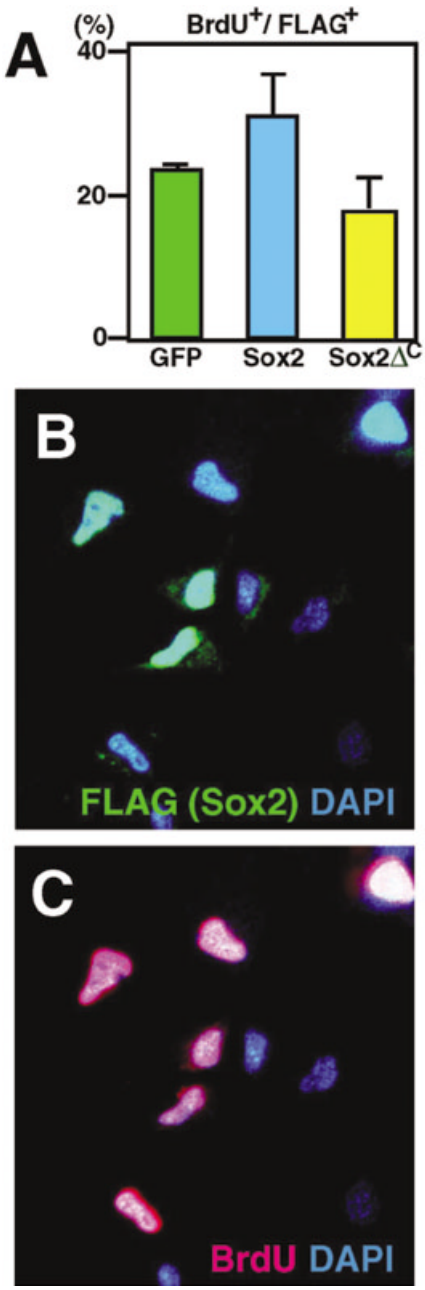

Fig. 7. The effect of *Sox2* misexpression on proliferation of cultured neural crest cells. **A:** Misexpression of full-length *Sox2* moderately promotes cell proliferation. Transfection of *Sox2*$^{\Delta C}$ has no significant effect on proliferation. One and a half hours of bromodeoxyuridine (BrdU) pulse-labeling was performed 24 hr after transfection. Every experiment was repeated for five times, and the average is shown. To obtain each value, more than 200 FLAG-positive cells were examined, and the proportion of BrdU-positive, FLAG-positive cells/FLAG-positive cells was obtained. Error bars indicate standard deviation. **B,C:** An example of full-length *Sox2*-transfected cells with BrdU-labeling. DAPI, 4′,6-diamidine-2-phenylidole-dihydrochloride.

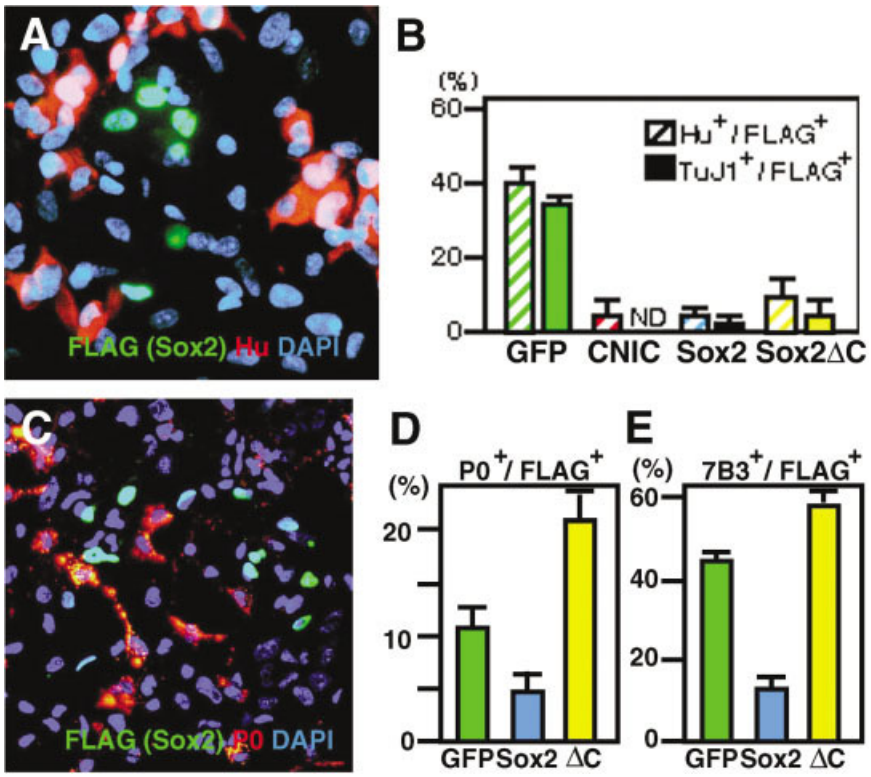

Fig. 8.

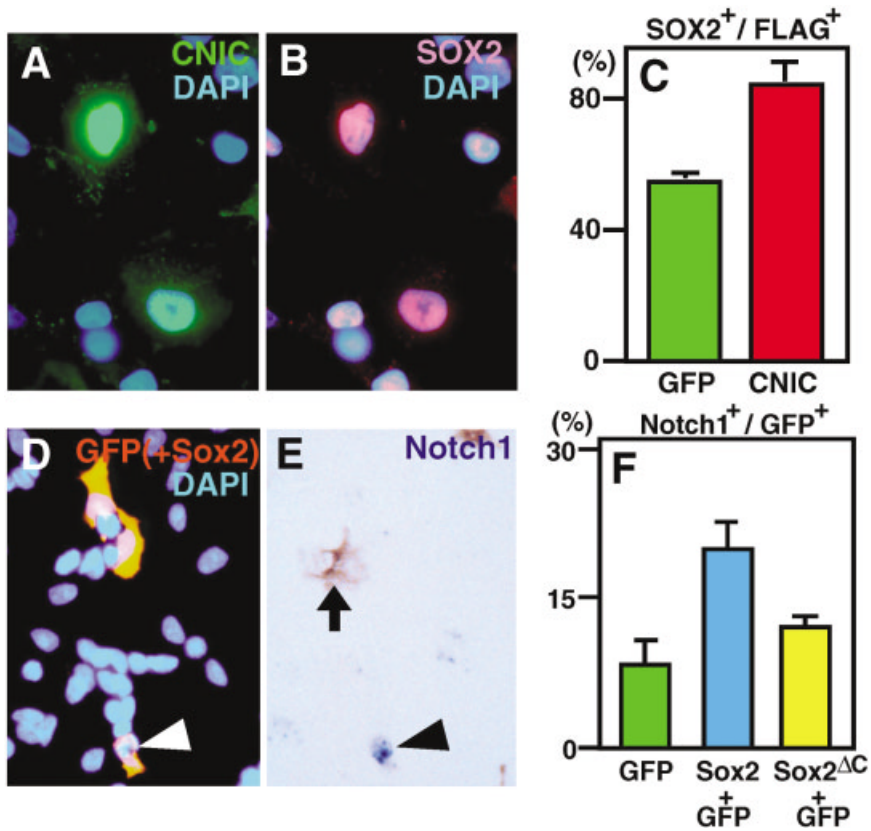

Fig. 9.

Sox2 Affects Proliferation and Differentiation in Culture

Although in vivo analyses of *Sox2* constructs suggested the involvement in PNS development, because *Sox2* misexpression also affected neural crest formation, the observed phenotypes of the transfected cells in the developing PNS might be secondary to the earlier effects. Thus, to assess the function of *Sox2* in the late phase of neural crest development, we performed misexpression analyses in cultured trunk crest cells (Wakamatsu and Weston, 1997; see also Experimental Procedures section). Neural tubes were taken from stage 12–14 quail embryos and cultured for 13 hr. Then, the crest cell outgrowth was transfected and replated.

Because the *Sox2* expression domain in the developing DRG coincides with the domain of active proliferation (Wakamatsu et al., 2000), we first examined the effect of the transgenes on proliferation (Fig. 7A–C). Twenty-four hours after transfection, BrdU pulse-labeling was performed, and a proportion of BrdU-positive cells in FLAG-positive transfected cells was examined. Overexpression of full-length *Sox2* promoted proliferation (31%), compared with the *GFP*-transfected negative control (23%). This increase was comparable to the result when cells were transfected with the activated form of *Notch1*, examined under corresponding condition (37%; Wakamatsu et al., 2000, see below). Cultures in which $Sox2^{\Delta C}$ was misexpressed showed no significant increase of proliferation (19%).

Next, to assess the effect of these transgenes on neuronal differentiation, we examined expression of neuronal markers 72 hr after transfection (Fig. 8A,B). Overexpression of full-length *Sox2* dramatically decreased the proportion of neuronal cells (5% Hu-positive, 2% β-TUBULIN–positive), compared with the *GFP*-transfected negative control (41% Hu-positive, 37% β-TUBULIN–positive). This decrease was identical to the result when the activated form of *Notch1* was transfected (5% Hu-positive; Wakamatsu et al., 2000; see below). Consistent with the in vivo results (see above), $Sox2^{\Delta C}$ expression also decreased the proportion of neuronal cells (12% Hu-positive, 6% β-TUBULIN–positive), nearly as much as did the full-length *Sox2*.

Seventy-two hours after transfection, very few *Sox2-En*^{R}–expressing cells were found, although transfected FLAG-immunoreactive cells could be observed 24 hr after transfection. Failure to detect such cells is probably due to induced cell death, as observed in vivo (see above). Likewise, 72 hr after transfection, no $Sox2^{\Delta N}$-expressing cells were found. Because $Sox2^{\Delta N}$ did not induce cell death in vivo, this loss of transfected cells might have a different cause. For example, because misexpression of full-length *Sox2* appeared to increase cohesiveness of crest cells both in vivo (Fig. 6B,C) and in culture (data not shown), this finding might be due to the detachment of $Sox2^{\Delta N}$-transfected cells from culture dish. Nevertheless, the loss of transfected cells prevented us from testing the effects on differentiation with those constructs.

To test whether *Sox2* and/or $Sox2^{\Delta C}$ inhibited general differentiation or promoted glial differentiation as an alternative fate, we examined the expression of a glial-specific cell-surface protein, P0 (Fig. 8C,D). Full-length *Sox2* appears to inhibit glial differentiation (5%), compared with the negative control with *GFP* (11%) ($P < 0.005$ relative to *GFP*-transfected control by Student's *t*-test). This result was similar to that when the activated form of *Notch1* was transfected (8%; Wakamatsu et al., 2000). Of interest, $Sox2^{\Delta C}$ misexpression showed an increase in the proportion of P0-positive glial cells (21%; $P < 0.002$ relative to *GFP* transfection, Fig. 8D). The effect of $Sox2^{\Delta C}$ on glial differentiation could be abrogated by the cotransfection of the constitutively active form of *Notch1* (5% P0-positive). To examine whether *Sox2*-transfected cells remained undifferentiated, we further examined the expression of 7B3 antigen, which recognizes intermediate filament protein expressed low in undifferentiated crest cells but up-regulated in glial cells (Henion et al., 2000). *Sox2*-transfected cells showed decreased 7B3

Fig. 8. Effects of *Sox2* constructs on the differentiation of cultured crest cells. Cells were fixed 72 hr after transfection and stained with anti-FLAG and differentiation markers. Every experiment was repeated for four times. To obtain each value, more than 200 FLAG-positive cells were examined, and the proportion of differentiation marker-positive, FLAG-positive cells/FLAG-positive cells was obtained. Error bars indicate standard deviation. **A,B:** Both full-length *Sox2* and $Sox2^{\Delta C}$ constructs inhibit neuronal differentiation. FLAG-SOX2-positive cells (green) do not express a neuronal marker Hu (red) and segregate from neuronal cells (A). **C,D:** Full-length *Sox2* misexpression decreases the proportion of P0-positive glial cells. $Sox2^{\Delta C}$ misexpression moderately permits glial differentiation. FLAG-SOX2–positive cells (green) do not express a glial surface antigen P0 (red, C). **E:** Misexpression of full-length *Sox2* decreases the proportion of 7B3 antigen-positive glial cells. DAPI, 4′,6-diamidine-2-phenylidole-dihydrochloride; GFP, green fluorescent protein.

Fig. 9. NOTCH activation induces SOX2 expression. **A–C:** NOTCH activation promotes SOX2 expression. A FLAG-tagged activated form of chicken *Notch1*, $CNIC^{\Delta C89}$, or *GFP* were transfected into cultured crest cells, and endogenous SOX2 expression was examined 3 days after transfection. Every experiment was repeated for three times. To obtain each value, more than 200 FLAG-positive cells were examined, and the proportion of SOX2-positive, FLAG-positive cells/FLAG-positive cells was obtained. Error bars indicate standard deviation. A,B: An example of $CNIC^{\Delta C89}$-transfected cells expressing SOX2. $CNIC^{\Delta C89}$ is FLAG-tagged and localizes in the nuclei. **D–F:** The full-length SOX2 weakly promotes endogenous *Notch1* mRNA expression. $Sox2^{\Delta C}$ does not have a significant effect on *Notch1* expression. Because FLAG-epitope could not be detected after in situ hybridization, to detect *Sox2*-transfected cells, *GFP* was cotransfected and GFP was detected by anti-GFP antibody. Every experiment was repeated for three times. To obtain each value, more than 200 FLAG-positive cells were examined, and the proportion of *Notch1*-positive, GFP-positive cells/GFP-positive cells was obtained. Error bars indicate standard deviation. D,E: An example of *Sox2* and *GFP* cotransfected cells. One of three cells in the picture coexpresses *Notch1* mRNA (arrowhead). An arrow indicates pigmented melanocyte, which is not transfected. DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride); GFP, green fluorescent protein.

immunoreactivity (13%, Fig. 8E), compared with *GFP*-transfected cells (45%), confirming the inhibition of general differentiation, while $Sox2^{\Delta C}$-misexpression weakly increased the proportion of 7B3-positive cells (59%).

In summary, full-length *Sox2* inhibits both neuronal and glial differentiation, and promotes proliferation. In contrast, $Sox2^{\Delta C}$ inhibits neuronal differentiation and promotes or permits glial differentiation but does not promote proliferation.

Sox2 Expression Is Induced by NOTCH Signaling

As described above and as previously reported (Wakamatsu et al., 2000), the domain of *Sox2* and *Notch1* expression overlaps in the periphery of developing DRG. Furthermore, inhibition of both neuronal and glial differentiation and promotion of proliferation by full-length *Sox2* misexpression are similar to the results by misexpression of constitutively active *Notch1* (see above, and Wakamatsu et al., 2000). We hypothesized, therefore, that *Sox2* expression might be induced by NOTCH activation. Transfection of $CNIC^{\Delta C89}$, an activated form of chicken *Notch1* (see Wakamatsu et al., 1999, 2000), showed a significant increase of SOX2-positive cells (85%), compared with the *GFP*-transfected control (54%; Fig. 9A–C; $P < 0.0002$ relative to *GFP*-transfected control). We also examined the effect of *Sox2* constructs on *Notch1* mRNA expression (Fig. 9D–F). Misexpression of full-length *Sox2* indeed increased the proportion of *Notch1*-positive cells (19%), compared with the negative control (8%) ($P < 0.003$ relative to *GFP*-transfected control). However, the proportion of such cells was low, suggesting that *Sox2* expression was not sufficient to promote and/or maintain *Notch1* mRNA expression. $Sox2^{\Delta C}$ did not significantly change the proportion of *Notch1*-positive cells (12%; $P > 0.2$ relative to *GFP* transfection, Fig. 9F). These observations suggest that *Sox2* regulates differentiation of crest-derived DRG cells downstream of NOTCH signaling.

DISCUSSION

Sox2 Is a Negative Regulator of Neural Crest Formation

In this study, we showed that *Sox2* expression is down-regulated in premigratory and migratory neural crest cells (see also Endo et al., 2002) and that misexpression of *Sox2* in the neural fold inhibits neural crest formation. These data indicate that *Sox2* inhibits neural crest specification within the neural plate/neural tube. Our results superficially conflict with previous observations in *Xenopus* (Mizuseki et al., 1998; Kishi et al., 2000). For example, in the animal caps taken from *Sox2* and *FGF* mRNA-injected embryos, *Xslug* expression is induced, along with other neural markers (Mizuseki et al., 1998). Dominant-negative *Sox2* constructs reduce *Xslug* expression along with other neural markers (Kishi et al., 2000). It is important to note that such an increase and a decrease of neural crest in these assays are always accompanied by the increase and the decrease of neural tissue, respectively. It has been known that the mutual interaction of neural plate and epidermal ectoderm is important for the neural crest induction. Thus, the effects observed in *Xenopus* system can be interpreted by a secondary effect of the increase and the decrease of neural tissue-derived signal(s). In any case, *Sox2* appears to inhibit neural crest formation in more direct manner at the stage after the neural differentiation, at least in avian embryos. Still, it is likely that *Sox2* acts indirectly on *Slug* expression, because *Sox2* inhibits *Slug* expression as a transcriptional activator, as revealed by a misexpression of *Sox2-VP16* fusion. Consistent with this notion, *Sox2* interferes with *Slug* induction by BMP4. Nevertheless, *Sox2* appears to restrict neural crest formation in the lateral neural plate. As shown in our previous study, NOTCH signaling in the epidermal ectoderm cell autonomously inhibits neural crest formation (Endo et al., 2002). Thus, both *Sox2* and NOTCH signaling act on confining the area of neural crest induction to the junction of neural plate and epidermal ectoderm.

Sox2 Regulates Differentiation of Crest-Derived Cells in Developing PNS

In nascent DRGs, it has been shown that the neuroglial progenitors segregate from neuronal cells, localize in the periphery of the DRG, and actively proliferate, revealed by BrdU-labeling experiments (Wakamatsu et al., 2000). *Sox2* is preferentially expressed in such peripheral cells of the DRG, which later generate neurons and glia. *Sox2* expression is subsequently restricted in glial sublineages and is never expressed in neurons. Such expression patterns suggest the involvement of *Sox2* in the proliferation of undifferentiated peripheral cells and the differentiation of neurons and glia. Consistently, full-length *Sox2* misexpression promotes proliferation, and inhibits neuronal differentiation. Despite endogenous expression of *Sox2* in glial sublineages, however, *Sox2* overexpression inhibits glial differentiation, as monitored by expression of a glial marker P0. This result seems paradoxical. However, misexpression of $Sox2^{\Delta C}$ permits glial differentiation, while still preventing neuronal differentiation. Thus, the multiple actions of *Sox2* in regulating differentiation of crest-derived PNS cells can be distinguished by our deletion studies.

That SOX2 appears to require a transcriptional partner for the activation of down-stream target genes (reviewed in Kamachi et al., 2000) may account for some of this complexity. For example, SOX2 activates lens-specific transcription of *δ1-crystallin* gene in combination with PAX6, and such activity diminishes when the C-terminal region of SOX2 is removed (Kamachi et al., 1995, 1998, 2001). Although the C-terminal end appears to be the authentic transactivation domain, the proximal region of C-terminal sequence appears to be involved in the interaction with PAX6. It is suggested, therefore, that SOX2 requires a partner(s) for transcriptional activation, and the interaction with such partner may be mediated by the C-terminal half of SOX2 (Kamachi et al., 2000). This inference is in agreement with the previous observation that, in *Xenopus*, SOX2-mediated neural dif-

ferentiation can be blocked by overexpression of the C-terminal half, which resembles our *Sox2*$^{\Delta N}$ construct. SOX2 also activates an FGF4 enhancer through the interaction with OCT3 (Yuan et al., 1995). In this case, however, SOX2 can interact with OCT3 through its HMG-box DNA-binding domain, and such interaction does not require the C-terminal region (Ambrosetti et al., 1997). Therefore, SOX2 has distinct modes of transcriptional activation, depending on which domain of SOX2 is involved and what kind of partner interacts with SOX2. In this study, we could dissociate the function of SOX2 by transfecting deletion constructs. Thus, *Sox2*$^{\Delta N}$ transfection in vivo promotes neuronal differentiation, while *Sox2*$^{\Delta C}$ has no effect on proliferation, inhibits neuronal differentiation, and permits glial differentiation. Because *Pax6* is not expressed in the developing PNS (Y. Wakamatsu, unpublished observations) and *Oct3/4* homolog is reportedly not present in the chick genome (Soodeen-Karamath and Gibbins, 2001), SOX2 probably acts along with other transcriptional partner(s) in the PNS development. To understand the modes of *Sox2* function further, the various binding partners of SOX2 in crest-derived cells should be determined.

Sox2 Regulates Glial Differentiation of Crest-Derived Cells in the Downstream of NOTCH Signaling

Expression of *Notch1* and *Sox2* overlaps in undifferentiated crest-derived cells in the periphery of developing ganglia (see also Wakamatsu et al., 2000). Both *Sox2* misexpression and activation of NOTCH signaling inhibit neuronal differentiation and promote proliferation. Our misexpression studies show that NOTCH activation is sufficient to maintain and/or trigger endogenous SOX2 expression. Thus, it is most likely that *Sox2* functions downstream of NOTCH signaling. It has been argued whether NOTCH activation directly promotes glial differentiation. In our previous study, transfection of activated *Notch1* in cultured trunk crest cells moderately decreased the proportion of P0 expressing glial cells. Moreover, cotransfection of *Sox2* and activated *Notch1*-inhibited glial differentiation to the same extent as each construct alone. However, we previously suggested that constitutive activation of NOTCH signaling may interfere with glial terminal differentiation and that transient NOTCH activation may trigger proper gliogenesis (Wakamatsu et al., 2000). Consistently, it is reported that transient NOTCH activation with a NOTCH–ligand–Fc fusion protein in cultured mammalian crest-derived cells promotes glial differentiation (Morrison et al., 2000). Therefore, transient NOTCH activation may be able to release crest cells from the inhibitory effect of *Sox2* on glial differentiation. Alternatively, additional signal(s) may be required for proper glial differentiation. Because *Sox2*$^{\Delta C}$ construct increases the proportion of P0-positive cells in crest culture, changes in the composition of SOX2 partner(s) may occur for glial differentiation, and the C-terminal sequence of SOX2 is dispensable for interaction(s) with such partner(s).

Sox Genes in Neural Crest Development

While our results suggest an inhibitory effect of *Sox2* on neural crest formation, other Sox genes, such as *Sox9* and *Sox10*, have been shown to positively regulate neural crest formation in *Xenopus* embryos (Spokony et al., 2002; Honore et al., 2003). One possible way of *Sox2* to inhibit neural crest formation may be caused by a competition for the target binding sites. However, because *Sox2* acts as an activator in this case, and as *Sox9/10* activates target genes in general (Peirano et al., 2000; Britsch et al., 2001; Stolt et al., 2002), it is more likely that *Sox2* and *Sox9/10* have different targets of their own. While *Sox2* and *Sox10* have opposing effects on neural crest formation, these genes have a similar function on the PNS development. While, as recently shown, *Sox10* is required for glial differentiation (Britsch et al., 2001; Paratore et al., 2001), it also prevents neuronal differentiation and maintains multipotency of autonomic progenitors (Kim et al., 2003). Because expression of *Sox10* in crest-derived PNS cells appears to overlap with that of *Sox2* (Cheng et al., 2000; Honore et al., 2003; Kim et al., 2003), *Sox10* and *Sox2* may regulate cell differentiation in the developing PNS in concert.

Taken together, we conclude that *Sox2* is involved in multiple steps of neural crest development in avian embryo. To understand further the role of *Sox2* in neural crest development, it will be essential to identify *Sox2* targets and partners.

EXPERIMENTAL PROCEDURES

Experimental Animals

Fertilized chicken and Japanese quail (*Coturnix coturnix japonica*) eggs were obtained from the Oregon State University, Animal Sciences Department (Corvallis, OR), and Sendai Jun-ran, Sendai. Embryos were staged according to Hamburger and Hamilton (1951).

Antibodies and Immunologic Staining

16A11 anti-Hu (mouse IgG2b; Marusich et al., 1994), MEBL-1 anti-MMP115 (mouse IgG1; Kitamura et al., 1992, Wakamatsu et al., 1998), 1E8 anti-P0 (mouse IgG1; Bhattacharyya et al., 1991), 62.1E6 anti-SLUG (mouse IgG; Liem et al., 1995), HNK1 (mouse IgM; Tucker et al., 1988), and anti-SOX2 (rabbit polyclonal; Kamachi et al., 1998; Wakamatsu et al., 2000) antibodies were used as described previously. Anti-BrdU (mouse IgG1, Roche), TuJ1 anti–neuron-specific type III β-TUBULIN (mouse IgG2a, BAbCO), M2 anti-FLAG (mouse IgG1, Sigma), anti-FLAG (rabbit polyclonal, Zymed), anti-GFP (mouse IgG, Clontech), and Y11 anti-HA tag (rabbit polyclonal, Santa Cruz) antibodies were purchased from their commercial sources. Fluorochrome-conjugated secondary antibodies were purchased from Southern Biotechnologies (anti-mouse IgM–fluorescein isothiocyanate (FITC), anti-mouse IgG1-FITC, anti-mouse IgG2b–tetrarhodamine isothiocyanate (TRITC)), Chemicon (anti-mouse IgG-cy3),

Cappel (anti-rabbit IgG-cy3), and Jackson (anti-rabbit IgG-FITC, anti-mouse IgG-TRITC, anti-mouse IgG-FITC).

Immunologic staining of fixed cells and tissues was performed as described previously (Wakamatsu et al., 1993). In brief, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 hr, except for anti-SLUG antibody, which requires shorter fixation (20–30 min). The 6- to 7-μm cryosections were placed on VectaBond (Vector) -coated slides, and blocking was performed with 3% bovine serum albumin in Tris-buffered saline containing 0.1% of Tween20 (TTBS). Sections were incubated with primary antibodies overnight at 4°C, and washed in TTBS. Sections were then incubated with secondary antibodies for 1 hr at room temperature and were washed in TTBS, followed by a nuclear-staining with DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride). Sections were finally mounted with VectaShield (Vector). For anti-P0 staining of cultured cells, crest cells obtained from quail trunk neural tubes were incubated in culture with medium containing a primary antibody at 37°C for 1 hr. After washing in the medium, cultures were further incubated with a medium containing cy3-conjugated anti-mouse IgG for 1 hr at 37°C. After washing, cells were fixed in 4% paraformaldehyde in PBS for 10 min and stained with anti-FLAG antibody and DAPI.

In Situ Hybridization

The full coding region of quail *Sox2* was amplified from oligo-dT primed day 3 embryo cDNA by polymerase chain reaction (PCR). Primer sets were designed according to the chicken sequence (Kamachi et al., 1998). Quail *Notch1* probe was described previously (Wakamatsu et al., 2000). Quail *Slug* was cloned during the search for neural crest specific genes (Y. Wakamatsu, unpublished observations; see also Cheng et al., 2000; Endo et al., 2002). In situ hybridizations in whole-mount preparation and on section were performed as described previously (Wakamatsu and Weston, 1997). In situ hybridizations with chicken probes in quail tissues gave identical results with the same probes in chicken tissues.

Construction of Expression Vectors

Full-coding, N-terminal half (corresponding to amino acid 1-120), and C-terminal half (corresponding to amino acid 114-311) of quail *Sox2* sequence, as well as quail *Slug* ORF sequence were PCR amplified, and inserted into *pyDF30* plasmid for FLAG-epitope tagging (Wakamatsu and Weston, 1997). A plasmid containing *En2* repressor domain was obtained from Dr. Nakamura (Mastunaga et al., 2000). Quail *Sox2* cDNA was cut with *Kas*I, and the *Sma*I fragment of En2 was connected in frame. Such tagged sequences were transferred into *pmiwSV* (Wakamatsu and Weston, 1997) for expression studies. All four *Sox2* constructs resulted in nuclear localization of anti-FLAG immunoreactivity in transfected cells, although some mutant proteins also appeared in the cytoplasm at lower levels (not shown). *Sox2-VP16* fusion construct was kindly provided by Dr. Kamachi (Kamachi et al., 1999). FLAG-tagged expression vectors of *GFP* and *CNIC*$^{\Delta C89}$ have been described previously (Wakamatsu and Weston, 1997; Wakamatsu et al., 1999, 2000).

Electroporation to Cultured Quail Embryos and Chicken Embryos In Ovo

Whole-embryo culture and electroporation of expression vectors into the developing neural fold was previously described (Endo et al., 2002). In brief, stage 5–6 quail embryos were cultured on collagen-coated membrane. DNA solution (5 μg/μl in PBS containing 0.025% Fast Green) was carefully placed on the right prospective neural fold, and three rectangular pulses of 7 V, 25 msec, with 200-msec intervals were applied by the electorporator (CUY21; Neppa Gene). After electroporation, embryos were cultured in F12-based medium for 9 or 24 hr at 38°C.

For the electroporation of trunk crest cells, chicken eggs were windowed, and DNA solution was injected into the lumen of the neural tube at the last five-somite level of stage 12–14 embryos. Electrode and condition of pulse were as described previously (five rectangular pulses of 25 V, 50 msec, with 250-msec intervals; Funahashi et al., 1999). Transfected embryos were harvested 48 hr later.

Neural Plate Explant Culture

Electroporation was performed to the neural plate of stage 6–7 quail embryos as described above. Then, an intermediate portion of the neural plate at the level of midbrain was excised with tungsten needle along with underlying mesoderm and endoderm. Neural plate tissue was isolated by pancreatin treatment and cultured in a culture dish coated with fibronectin (Sigma) and filled with Ham's F12 culture medium containing 3% fetal bovine serum. To induce neural crest cells, culture medium was supplemented with BMP4 (20 ng/ml, R & D systems) and N2 supplement (1/100 dilution, Invitrogen; see also Liem et al., 1995; Selleck and Bronner-Fraser, 2000). After 18 hr of culture, cells were fixed, and processed for immunostaining as described above.

Neural Crest Culture and Transfection

Primary culture of quail trunk crest cells and BrdU pulse-labeling were performed as described previously (Marusich et al., 1994; Wakamatsu and Weston, 1997). Stage 12–14 quail neural tubes were prepared as described previously (Loring et al., 1981; Glimelius and Weston, 1981). Neural tube explants were cultured in neurogenesis-permissive medium (Henion et al., 1995; Ham's F12 supplemented with 15% fetal bovine serum and 4% chicken embryo extract) for 12–13 hr. The cultured cells were then transfected with Fugene6 (Roche) according to the provided protocol, and subsequently, the neural tubes were removed. As for cotransfection of *Sox2* and *GFP*, to ensure the

expression of SOX2 in GFP-positive cells, plasmid DNAs for *Sox2* and *GFP* were mixed at the ratio of 3:1. Neural crest cells including transfectants were then dissociated with trypsin–ethylenediaminetetraacetic acid. Harvested crest cells were re-plated at 4×10^3 cells/10 mm diameter Sylgard well (Dow Corning; Marusich and Weston, 1992) in the neurogenesis-permissive medium.

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