

Requirement of *Sox2*-mediated signaling for differentiation of early *Xenopus* neuroectoderm

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SUMMARY

From early stages of development, *Sox2*-class transcription factors (*Sox1*, *Sox2* and *Sox3*) are expressed in neural tissues and sensory epithelia. In this report, we show that *Sox2* function is required for neural differentiation of early *Xenopus* ectoderm. Microinjection of dominant-negative forms of *Sox2* (*dnSox2*) mRNA inhibits neural differentiation of animal caps caused by attenuation of BMP signals. Expression of *dnSox2* in developing embryos suppresses expression of *N-CAM* and regional neural markers. We have analyzed temporal requirement of *Sox2*-mediated signaling by using an inducible *dnSox2* construct fused to the ligand-binding domain of the glucocorticoid

receptor. Attenuation of *Sox2* function both from the late blastula stage and from the late gastrula stage onwards causes an inhibition of neural differentiation in animal caps and in whole embryos. Additionally, *dnSox2*-injected cells that fail to differentiate into neural tissues are not able to adopt epidermal cell fate. These data suggest that *Sox2*-class genes are essential for early neuroectoderm cells to consolidate their neural identity during secondary steps of neural differentiation.

Key words: Dominant-negative, Neural differentiation, Neuroectoderm, *Sox*, *Xenopus*

INTRODUCTION

Recent molecular studies on neural induction have identified key molecules that regulate neural differentiation of embryonic ectoderm. In *Xenopus*, neural inducers such as Noggin, Chordin and Follistatin are secreted by the Spemann organizer and can promote neuralization of animal cap ectoderm (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1995). These factors inactivate BMP4 by binding to it in the extracellular space. The role of BMP4 is to inhibit neural differentiation and to promote epidermal differentiation of ectoderm (Wilson and Hemmati-Brivanlou, 1995; Sasai et al., 1995). BMP4 signaling through BMP receptors is mediated by the Smad system as well as possibly by the TAB/TAK system (Massague, 1998; Shibuya et al., 1998). As a consequence, downstream genes such as *GATA1* and *Msx1* are induced as primary response genes. Overexpression of *GATA1* and *Msx1* can also suppress neuralization of ectoderm caused by neural inducers (Xu et al., 1997; Suzuki et al., 1997).

Several genes that promote neural differentiation have also been isolated. *Xenopus* homologues of *Drosophila odd-paired* (*Zic*-related factors) are expressed in the neuroectoderm of early gastrulae and can initiate neural differentiation of animal cap ectoderm when overexpressed (Nakata et al., 1997; Mizuseki et al., 1998a). A *Zic1*-related zinc finger factor, called opl, is reported to promote neuronal differentiation when

combined with Noggin (Kuo et al., 1998). *SoxD* is a distant member of the *Sox* gene family and its transcription is regulated by Chd/BMP signals (Mizuseki et al., 1998b). Regulation of *SoxD* is positively controlled by *Zic1* and negatively by *GATA1* and *Msx1*. *SoxD* transcripts first appear during late blastula stages in a pan-ectodermal fashion. *SoxD* expression becomes limited to the neuroectoderm around the time of mid-gastrulation and remains pan-neural during the rest of embryogenesis. Microinjection of *SoxD* mRNA causes neural differentiation of ectoderm both in vivo and in the animal cap (Mizuseki et al., 1998b). A POU-class homeobox gene, *Xlpou2*, which is expressed in the neuroectoderm of frog gastrulae, is also capable of inducing neuralization in a similar manner to *Zic* and *SoxD* (Witta et al., 1995). Another group of genes acting in early neurogenesis is vertebrate homologues of *Drosophila* proneural and neurogenic genes (Ma et al., 1996; Lee, 1997). In *Xenopus*, *Xngnr1* (a neurogenin-related bHLH factor) is expressed in the primary neuron precursors and can induce neuronal differentiation in vivo and in the animal cap. Other bHLH factors (e.g., *Xash3*, *NeuroD*, *Xath3*) are also expressed in early neural plate and seem to play positive roles in parallel with or downstream of *Xngnr1*.

The *Sox* gene family encode Sry-related transcription factors containing an HMG DNA-binding domain (Pevny and Lovell-Badge, 1997). The *Sox* family are subdivided into several subfamily groups on the basis of structural similarity (Wegner,

1999). *Sox2* and its closely related genes (*Sox1* and *Sox3*) are classified as subfamily group B genes, and share more than 90% amino acid residue identities in their DNA-binding domains. These values are significantly higher than those found among DNA-binding domains of different subfamily groups. For instance, *Sox9*, which belongs to group E, shares about 60% amino acid identity to *Sox2* in the DNA-binding domain. *Sox1*, *Sox2* and *Sox3* are expressed in early neuroectoderm of many vertebrate embryos and are also expressed in the forming lens (Wegner, 1999 and references therein). *Sox2*-class factors bind to the promoter region of a *crystallin* gene and transactivate this gene when working with lens-specific cofactors (Kamachi et al., 1995). Although the *Sox1* gene knockout has been shown to cause defects in lens maturation (Nishiguchi et al., 1998), little is yet known about the role of *Sox2*-class genes in early neural differentiation.

Two recent studies have reported possible involvement of *Sox2*-class factors in early steps of neural differentiation. In *Xenopus*, a combination of *Sox2* and bFGF can promote posterior neural differentiation in the animal cap. Neither exposure to FGF nor overexpression of *Sox2* alone can initiate neural differentiation, suggesting that *Sox2* can change the responsiveness of the ectoderm to FGF signaling (Mizuseki et al., 1998a). Mammalian cell culture studies also suggested a possible role of a *Sox2* subfamily member. Neural differentiation of P19 embryonic carcinoma cells requires two distinct signals; exposure to retinoic acid and cell aggregation. Overexpression of *Sox1* can substitute for the requirement of retinoic acid (Pevny et al., 1998).

To further understand the roles of *Sox2*-class genes in early neural development, we attempted to perform loss-of-function studies by using dominant-negative forms of *Sox2* (*dnSox2*). Generally, the dominant-negative

approach, as compared to the gene disruption strategy, is expected to have an advantage in a system where multiple closely related genes are present. In the case of *Sox2*, at least three subfamily members (*Sox1*, *Sox2*, *Sox3*), are expressed in mouse and chick neuroectoderm (Uwanogho et al., 1995; Collignon et al., 1996; Kamachi et al., 1995). In *Xenopus*, *Sox3* transcripts are detected in developing nervous systems as well as in the maternal RNA pool (Penzel et al., 1997), although isolation of *Xenopus Sox1* has not yet been reported. By using *dnSox2*, we provide data showing that signaling mediated by *Sox2*-class factors is required for proper differentiation of *Xenopus* neuroectodermal cells into neural tissues.

MATERIALS AND METHODS

Plasmid construction and in vitro mRNA synthesis

To generate *Sox2BD(-)* construct, the *Sox2*-coding region lacking the most of the HMG domain (corresponding to residues 105-311) was amplified by PCR and inserted into the *EcoRI-XhoI* site of pCS2-NLS vector (Rupp et al., 1994). For FLAG-tagged *Sox2BD(-)*, the FLAG epitope sequence was added to *Sox2BD(-)* at the carboxyl terminus by PCR and the product was subcloned as above. For *Sox2-EnR* and *Sox9-EnR*, the cDNA fragments corresponding to the HMG domain (residues 31-121 and 99-189, respectively) obtained by PCR were first subcloned into pCS2-NLS vector. Then, *Drosophila engrailed* fragment containing the repressor region (amino acid residues 2-298; Conlon et al., 1996) was added to the carboxyl termini of the *Sox2* and *Sox9* HMG domains (pCS-NLS-*Sox2EnR* and pCS-NLS-*Sox9EnR*; the resultant constructs have a *EcoRI* and a *XbaI* sites at 5' and 3' ends, respectively). For *Sox2-GR*, *Sox3-GR*, *Sox9-GR* and *Sox2BD(-)-GR* plasmids, the *Xbra* cDNA fragment was removed

Fig. 1. Dominant-negative effects of *Sox2BD(-)* and *Sox2-EnR* on wild-type *Sox2*. (A) Structures of two *dnSox2* constructs. (Top) Wild type; (middle) *Sox2BD(-)*, lacking most of the DNA-binding HMG domain (black box). (Bottom) *Sox2-EnR*, the *Sox2* HMG domain was fused to the *Drosophila engrailed* repressor region (grey box) at the carboxyl terminus. (B-L) Neural differentiation of the animal caps was analyzed with the pan-neural *N-CAM* marker at stage 19. Injection of the *FGF4* expression plasmid (B) or control plasmid (inset) did not induce *N-CAM* expression in the explants. Coinjection of wild-type *Sox2* mRNA with the *FGF4* plasmid caused neural differentiation (C). This neural differentiation was suppressed by coinjecting *Sox2BD(-)* mRNA (D) or *Sox2-EnR* mRNA (G) in the caps injected with *Sox2* mRNA and the *FGF4* plasmid. This suppression was reversed by expressing *Sox2-GR* in the presence of 10 μ M Dex (E,H) but not in its absence (insets). *Sox9-GR* did not rescue *N-CAM* expression even with Dex (F,I). *SoxD* mRNA injection caused neural differentiation of animal caps (J). This neuralization was not inhibited by coinjection of *Sox2BD(-)* or *Sox2-EnR* mRNA (K,L). In each injection, the total amounts of RNA and DNA were made constant by adding an adequate amount of control *lacZ* mRNA and plasmid DNA. The injected amounts were 10 pg for *FGF4* plasmid, 100 pg for *Sox2* and *SoxD*, 400 pg for *Sox2BD(-)*, 50 pg for *Sox2-EnR*, 200 pg for *Sox2-GR* and 200 pg for *Sox9-GR* mRNA.

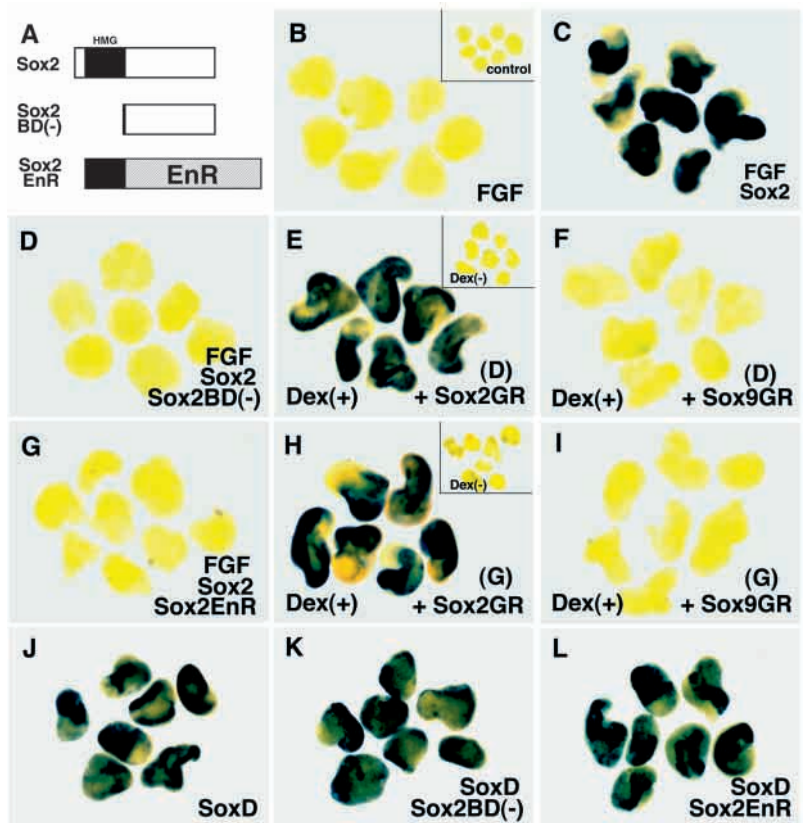
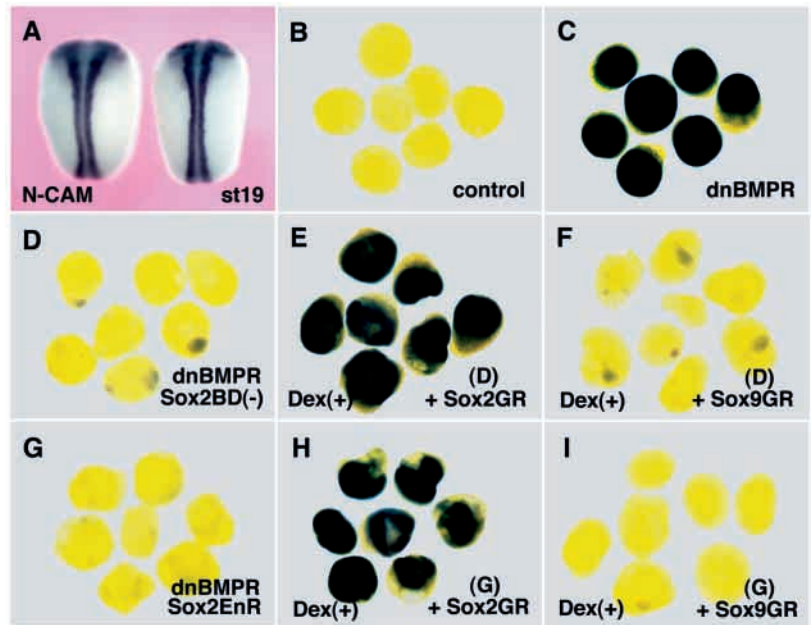


Fig. 2. Injection of *dnSox2* mRNAs suppresses neural differentiation of animal cap explants caused by *dnBMPR*. (A) Expression of N-CAM in normal embryos at stage 19, when the animal caps below were harvested. (B-I) Effects of *dnSox2* were examined with the N-CAM marker in the animal cap assay. Injection of *dnBMPR* (C) but not of control *lacZ* (B) mRNA induced N-CAM expression. The neural differentiation caused by *dnBMPR* was inhibited by coinjecting *Sox2BD(-)* mRNA (D) or *Sox2-EnR* mRNA (G). This inhibition was reversed by *Sox2-GR* coinjection in the presence of Dex (E,H) but not in its absence (not shown). The inhibitory effect of *Sox2BD(-)* or *Sox2-EnR* was not counteracted by *Sox9-GR* even with Dex treatment (F,I). In each injection, the total amounts of RNA were made constant by adding an adequate amount of control mRNA. The injected amounts of mRNA were 50 pg for *dnBMPR*, 400 pg for *Sox2BD(-)*, 50 pg for *Sox2-EnR*, 200 pg for *Sox2-GR*, 200 pg for *Sox9-GR*.



from pSP64T-*Xbra-GR-HA* (Tada et al., 1997) and replaced with the corresponding *Sox* cDNA inserts. The *FGF4*-expressing vector was generated by inserting the coding region of *Xenopus FGF4* (Isaacs et al., 1992) obtained by PCR into the *HindIII-PstI* site of pCSKA (Condie et al., 1990) which contains the *Xenopus* actin promoter. The cDNA fragments obtained by PCR were verified by sequencing. To synthesize sense mRNAs, the plasmids above were linearized with *NotI* (*Sox2BD(-)*, FLAG-tagged *Sox2BD(-)*), *BssHIII* (*Sox2-EnR*, *Sox9-EnR*), or *Sall* (*Sox2-GR*, *Sox3-GR*, *Sox9-GR*, *Sox2BD(-)-GR*) and subjected to *in vitro* transcription with SP6 polymerase (mMessage mMachine, Ambion).

Embryonic manipulations, RNA and DNA injection and dexamethasone treatment

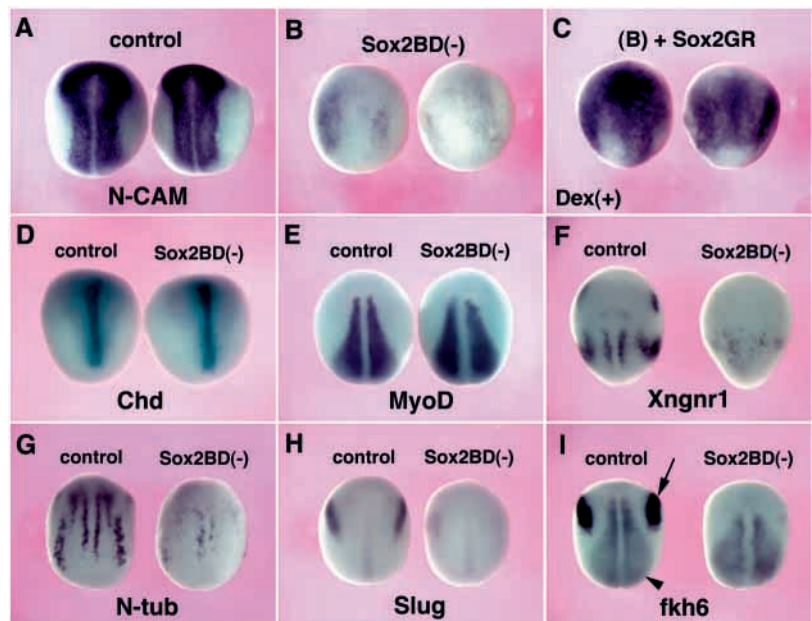
Xenopus laevis eggs were inseminated *in vitro* with testis homogenate. Embryos were kept in 0.1× Barth solution until indicated stages. Animal cap explants were prepared at stage 9 and cultured in 1× LCMR + 0.2% BSA unless mentioned. In experiments for neuralizing activity of *Sox2* with FGF4, animal caps were excised at stage 10.25. Synthetic mRNA and expression plasmids were injected into animal blastomeres of 8-cell embryos to overexpress the products

in the ectoderm. Dexamethasone was purchased from Sigma and the stock solution was made at 10 mM in ethanol. A final 10 μM concentration of Dex was added to the culture medium at the stage mentioned and was not removed until fixation. All the injection experiments were carried out at least twice and gave reproducible results. The statistical values given in the text were from one representative experiment. In each series of experiments, total amount of injected RNA and DNA was adjusted by adding neutral *lacZ* mRNA or control *lacZ* plasmid.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization analysis was performed as described (Chitnis et al., 1995) with minor modifications. For double-color staining, fixed embryos were incubated with one fluorescein-labeled and one DIG-labeled antisense RNA probes. The samples were then incubated with anti-fluorescein-POD and anti-DIG-AP antibodies (Boehringer) and stained sequentially with DAB (first) and with BM Purple (second). Signals in the animal caps were

Fig. 3. Injection of *Sox2BD(-)* inhibits neural differentiation *in vivo*. Whole-mount *in situ* hybridization analyses at the open neural plate stage (dorsal view; upside, anterior). (A-C) Both embryos were injected with synthetic mRNAs into each animal blastomere at the 8-cell stage: control *lacZ* mRNA (600 pg; A), *Sox2BD(-)* mRNA (400 pg + 200 pg *lacZ*; B) or *Sox2BD(-)* + *Sox2-GR* (400 pg + 200 pg; C). N-CAM expression in the neural plate was severely suppressed in *Sox2BD(-)*-injected embryos while *Sox2-GR* coinjection rescued N-CAM expression in the presence of Dex. (D-I) 400 pg of *Sox2BD(-)* mRNA (right) or control mRNA (left) were injected into each animal blastomere of the 8-cell embryo. Injection of *Sox2BD(-)* had little effect on the expression of the dorsal mesodermal markers *Chd* or *MyoD* (D,E) while the proneural gene *Xngnr1* (F), the neuronal marker *N-tubulin* (G), the neural crest markers *Slug* (H) and *fkh6* (I) were significantly suppressed. Note that the *fkh6* expression was inhibited in presumptive head crest regions (arrow) but not in posterior mesoderm (arrowhead).



photographed after counterstained with Bouin's fixative (yellow) and cleared in Murray's solution (Mizuseki et al., 1998a). Antisense RNA probes were prepared as described previously (Mizuseki et al., 1998b). The *MyoD* (Hopwood et al., 1989) and *HoxB9* (Cho et al., 1991) probes were synthesized from pM3 (kind gift of J. Gurdon; linearized with *Bam*HI) and pX4 (kind gift of C. Wright; linearized with *Xba*I; Wright et al., 1990) with SP6 and T3 RNA polymerases, respectively. The plasmids containing *Xenopus Zic2* (pBS-SK-*Zic2*) and *fkh6* (pBS-SK-*fkh6*) were isolated in our previous differential screen (Mizuseki et al., 1998a; GenBank accession number AB014461 and AB014661, respectively). The *Zic2* cDNA that we isolated contains a DNA sequence 95% identical to the *Zic2* sequence previously reported (Brewster et al., 1998) and is likely a different allele due to pseudotetraploidy of *Xenopus laevis*. The *Sox2* probe was prepared from the plasmid that contains *Sox2* 5'-UTR sequence which does not overlap with that of *Sox2BD(-)* (pCS2-*Sox2*5UTR). The Bluescript plasmids above and pCS2-*Sox2*5UTR were linearized with *Eco*RI and transcribed with T7 RNA polymerase.

Western blot and RT-PCR analysis

Western blot analysis was performed by using anti-FLAG M2 antibody and PVDF membrane filters (Immobilon, Millipore). Signals were visualized by using chemiluminescence reagents (Renaissance, NEN). Total RNA was prepared from stage 19 animal caps by the acid-phenol-GTC method (Trizol, Gibco-BRL) and was subjected to DNase I treatment to get rid of trace of genomic DNA (Message Clean Kit, GenHunter). RT-PCR analysis was performed as described previously (Mizuseki et al., 1998a).

Confocal microscopy

Embryos were injected with RNAs at the 8-cell stage and cultured until neurula stages. Embryos were fixed with MEMFA + 1% glutaraldehyde, embedded in 2% agarose gel and sectioned transversely with vibratome. Confocal images of injected tracer GFP and nuclear DAPI staining were collected on an Olympus BX-50 confocal microscope using appropriate filters.

RESULTS

Inhibition of *Sox2* activity by dominant-negative *Sox2* constructs

Two different candidate constructs for *Sox2* dominant-negative mutants were generated (Fig. 1A). The first construct (middle) lacks most of the DNA-binding HMG domain (*Sox2BD(-)*). In our previous study, a similar construct for another *Sox* gene, *SoxD*, worked efficiently as a dominant-negative mutant (*SoxDBD(-)*; Mizuseki et al., 1998b). In the second construct (bottom), the HMG domain of *Sox2* is fused with the engrailed repressor domain (*Sox2-EnR*). In *Xenopus* studies, a number of reports have shown that a chimeric construct of a DNA-binding and the engrailed repressor domains successfully functions as a dominant-inhibitory mutant (Conlon et al., 1996).

Effects of both mutants on wild-type *Sox2* activity were determined in animal cap assays. As previously reported (Mizuseki et al., 1998a), when wild-type *Sox2* and *FGF4* were overexpressed, the neural marker *N-CAM* was induced in animal cap explants (84%, $n=32$) (Fig. 1B,C). When *Sox2BD(-)* mRNA was coinjected with the wild-type *Sox2* mRNA, this induction was strongly suppressed (84%, $n=49$) (Fig. 1D).

We next asked if this suppression could be reversed by increasing wild-type *Sox2* mRNA. However, it proved that

higher doses of *Sox2* mRNA showed significant toxic effects. To avoid toxicity, we took advantage of a glucocorticoid receptor ligand-binding domain (*GR*) fusion strategy. It has been shown that activities of transcription factors fused with *GR* can be controlled by administration of dexamethasone (Dex; Kolm and Sive, 1995). In this study, the *GR*-fusion strategy was adopted to reduce the non-specific toxic effects by holding *Sox2-GR* inactive until required. In this manner, timing of activation of exogenous *Sox2* could be closer to that of endogenous *Sox2* (on and after stage 9) and the toxicity was avoided.

Sox2, *Sox2BD(-)* and *Sox2-GR* mRNA were injected into 8-cell-stage embryos. Animal caps were prepared at stage 10.25 and cultured with or without Dex. Coinjection of *Sox2-GR* clearly rescued expression of the neural differentiation marker *N-CAM* in animal caps cultured with Dex (80%, $n=44$; Fig. 1E) whereas in the absence of Dex it did not reverse suppression by *Sox2BD(-)* ($n=31$; Fig. 1E inset). Administration of Dex alone did not affect *N-CAM* expression ($n=23$; not shown). Injection of *Sox3-GR* rescued the neural differentiation suppressed by *Sox2BD(-)* in a similar manner to that observed for *Sox2-GR* (89%, $n=27$; not shown). In contrast, *Sox9-GR* failed to rescue the phenotype ($n=25$), showing that the effects are specific to *Sox2*-class members but not to *Sox* genes in general (Fig. 1F). These data demonstrated that *Sox2BD(-)* can function in the *Xenopus* ectoderm as a dominant-negative construct specific to *Sox2*-class factors.

Effects of *Sox2-EnR* were examined by the same approach as described above for *Sox2BD(-)*. Injection of *Sox2-EnR* mRNA suppressed induction of *N-CAM* expression caused by *Sox2* and FGF in animal caps (82%, $n=22$; Fig. 1G) while injection of *Sox9-EnR* did not ($n=37$; not shown). The suppression effect of *Sox2-EnR* was reversed by coinjecting with *Sox2-GR* in the presence of Dex (89%, $n=44$; Fig. 1H) but not in its absence ($n=40$; inset). *Sox9-GR* did not rescue the differentiation suppressed by *Sox2-EnR* ($n=40$; Fig. 1I). These results show that *Sox2-EnR* can also be used as a dominant-negative mutant.

A previous study has shown that another *Sox* family factor, *SoxD*, is involved in early neural differentiation (Mizuseki et al., 1998b). Therefore, we tested the effect of *dnSox2* constructs on the neuralizing activity of *SoxD* (Fig. 1J). Injection of *Sox2BD(-)* and *Sox2-EnR* mRNA (at the same amounts used above) did not significantly suppress *N-CAM* induction caused by *SoxD* (Fig. 1K,L; $n=30$ each). This suggests that *Sox2* signaling does not function downstream of *SoxD* in the regulation of neural differentiation.

Sox2-mediated signaling is required for neural differentiation of animal caps

We next asked if *Sox2*-mediated signaling is required for neural differentiation of animal caps caused by attenuated BMP signals. Overexpression of a dominant-negative BMP receptor (*dnBMPR*) in the animal cap shuts off endogenous BMP signals that are antineuralizing and initiates neural differentiation (Hemmati-Brivanlou and Melton, 1997). Fig. 2C shows the induction of *N-CAM* expression by *dnBMPR* injection in animal caps (100%, $n=32$). When *Sox2BD(-)* or *Sox2-EnR* was coinjected with *dnBMPR*, the animal caps failed to express the pan-neural marker *N-CAM* (Fig. 2D, 93%, $n=59$; Fig. 2G, 92%, $n=39$). This suppression of neural differentiation

was reversed by coinjecting *Sox2-GR* in the presence of Dex (Fig. 2E, 85%, $n=34$; Fig. 2H, 88%, $n=34$) but not in its absence ($n=32$ and 29 , respectively; not shown). *Sox9-GR* did not reverse the suppression by *dnSox2* (Fig. 2F and I; $n=36$ and 41 , respectively). These data demonstrate that signaling mediated by *Sox2* is required for animal cap ectoderm to differentiate into neural tissues.

Sox2 signaling is essential for neural development in vivo

The effects of *dnSox2* expression on neural development were tested in vivo. In the following in vivo studies, only *Sox2BD(-)* mRNA was utilized since *Sox2-EnR* mRNA injection, which worked well in animal cap assays, frequently caused exogastrulation when used in studies in vivo. It is difficult to evaluate neural formation in an embryo with exogastrulation because the interaction between ectoderm and mesoderm is impaired.

When 400 pg of *Sox2BD(-)* mRNA was injected into all the animal blastomeres of 8-cell embryos, *N-CAM* expression was inhibited in mid-neurula embryos (97%, $n=71$; Fig. 3A,B). *N-CAM* expression could be rescued by *Sox2-GR* injection with Dex (87%, $n=23$; Fig. 3C) but not without Dex ($n=21$; not shown), indicating that this suppression was not due to non-specific toxic effects of *Sox2BD(-)*. Dorsal mesodermal markers such as *Chordin* (*Chd*; Fig. 3D; Sasai et al., 1994) and *MyoD* (Fig. 3E; Hopwood et al., 1989) were intact ($n=28$, each), suggesting that the primary effect of *dnSox2* occurred in the ectoderm rather than in the mesoderm. Formation of primary neurons as depicted by *neurogenin* (*Xngnr1*) and *N-tubulin* (*N-tub*) was severely inhibited (Fig. 3F, 90%, $n=20$; Fig. 3G, 91%, $n=21$). Early neural crest markers such as *Slug* and *forkhead 6* (*fkh6*) were clearly suppressed (Fig. 3H, 90%, $n=29$; Fig. 3I, 100%, $n=23$). In the latter case, *fkh6* expression was suppressed only in the neural crest regions (arrow) but not in the posterior mesoderm (arrowhead).

Collectively, these results show that overexpression of *dnSox2* inhibits early neural development in general, including formation of the CNS, primary neurons and neural crest cells.

Sox2 signaling is required for expression of both anterior and posterior neural markers

We next asked if signaling mediated by *Sox2* is required for regional neural markers. This question is important since our previous studies have shown that *SoxD* is required for neural formation only in the anterior regions (Mizuseki et al., 1998b). Overexpression of *Sox2BD(-)* caused suppression of the anterior neural marker *Otx2* (Fig. 4A, arrow; 87%, $n=31$), with the posterior spinocaudal marker *HoxB9* unaffected (arrowhead). In contrast, overexpression of *Sox2BD(-)* significantly decreased both anterior and posterior neural markers: *Otx2* (forebrain; Fig. 4B, 92%, $n=36$), *Xanfl1* (anterior neural ridge and pituitary gland; Fig. 4C, 100%, $n=35$), *En2* (Midbrain-hindbrain boundary; Fig. 4D, 86%, $n=22$), *Krox20* (hindbrain; Fig. 4E, 88%, $n=24$) and *HoxB9* (spinal cord and posterior mesoderm; Fig. 4F, 91%, $n=21$). In Fig. 4F, *Sox2BD(-)* suppressed *HoxB9* expression only in the neural tissues (arrowhead) but not in the mesoderm (arrow). This observation is in good agreement with the ectoderm-specific expression of *Sox2*.

These data show that *Sox2*-mediated signaling is essential

for general neural differentiation as well as for regional specification.

Effects of dominant-negative Sox2 on expression of early neural markers

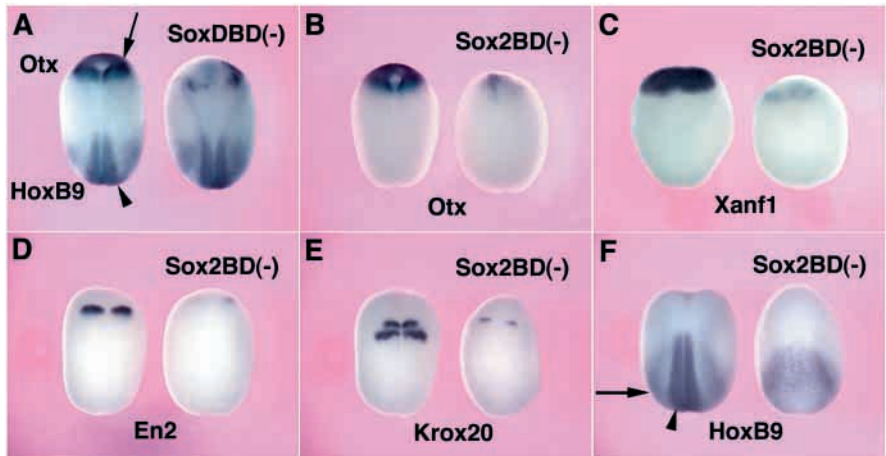
The data above examined the effects of *dnSox2* on neural markers in the neurula embryo or in the animal cap of the equivalent stage. Next, expression of earlier neural marker genes was determined in embryos injected with *dnSox2*. Effects of *dnSox2* were assayed in early neuroectoderm by using two early neural markers, *Xenopus Zic2* (Fig. 5A,C,E) and *Sox2* (Fig. 5B,D,F). In the latter case, the 5'UTR sequence not overlapping with injected *Sox2BD(-)* mRNA was used as a probe. At stage 11 (early gastrula), expression of *Zic2* and *Sox2* in embryos injected with *Sox2BD(-)* were not significantly suppressed as compared to that in the control embryos ($n=38$ and 30 , respectively), showing that early neural markers were intact in the early neuroectoderm (Fig. 5A,B). At stage 12 (late gastrula) and stage 14 (early neurula), expression of the two neural markers was reduced in the *Sox2BD(-)*-injected embryo significantly (Fig. 5C, 83%, $n=30$; Fig. 5D, 84%, $n=31$; Fig. 5E, 97%, $n=39$; Fig. 5F, 92%, $n=37$). To test if *Sox2BD(-)* protein accumulates at the gastrula stages, FLAG-tagged *Sox2BD(-)* mRNA, which has a similar dominant-negative activity to *Sox2BD(-)* mRNA, was injected into each animal blastomere of 8-cell embryos. Western blot analysis showed that translated FLAG-tagged *dnSox2* products were accumulated at high levels during early gastrula stages (Fig. 5G; stages 10 and 11), suggesting that the lack of *dnSox2* effects on early markers at stage 11 was not due to shortage of the gene product.

These results show two phases of effects of *dnSox2*. First, injection of *dnSox2* mRNA does not block formation of early neuroectoderm in the light of neural-specific gene markers (Fig. 5A,B). Second, initially formed neuroectoderm expressing *Zic2* and *Sox2* fails to maintain the expression of neural-specific genes when *Sox2*-mediated signaling is inhibited (Fig. 5C-F). This suggests that *Sox2* plays an essential role in maintenance or consolidation of neural fate of the cells in which initiation of neural differentiation has already taken place.

Sox2 signaling is necessary for late gastrula neuroectoderm to adopt neural fate

To determine the timing of *Sox2* requirement in early neural development, we made a chimeric construct of *Sox2BD(-)* with *GR* (*Sox2BD(-)-GR*; Fig. 6A), which was predicted to move into the nucleus under the control of exogenous Dex (Tada et al., 1997). In the absence of Dex, injection of *Sox2BD(-)-GR* mRNA per se did not affect *N-CAM* expression of the animal cap induced by *dnBMPR* ($n=29$; Fig. 6B,E). However, when Dex was applied to the culture saline from stage 9 (late blastula stage) onwards or from stage 12 (late gastrula stage) onwards, significant inhibition of neural differentiation was observed in each case (Fig. 6F, 100%, $n=23$; Fig. 6G, 82%, $n=51$). These data show that *Sox2*-mediated signaling is required for neural differentiation to proceed beyond late gastrula stages. Similar results were obtained when *Sox2BD(-)-GR* was applied in vivo. Overexpression of *Sox2BD(-)-GR* mRNA per se did not affect expression of *N-CAM* ($n=33$; Fig. 6I). When *Sox2BD(-)-GR*-injected embryos were treated with Dex from stage 9 or stage 12 onwards, clear decrease of *N-CAM* expression was observed (Fig. 6J, 88%, $n=25$; Fig. 6K, 85%,

Fig. 4. Injection of *Sox2BD(-)* inhibits expression of both anterior and posterior neural markers. Whole-mount in situ hybridization analyses were performed with regional neural markers in neurula embryos. (A) *SoxDBD(-)* mRNA (right) or control *lacZ* mRNA (left) was injected into each animal blastomere of the 8-cell embryo (400 pg of mRNA/cell). DIG-labeled *Otx2* and *HoxB9* probes were used simultaneously for double-label in situ hybridization. In the embryo injected with *SoxDBD(-)*, the expression of the forebrain marker *Otx2* (arrow) was suppressed whereas that of the spinocaudal marker *HoxB9* (arrowhead) was not reduced. (B-F) Embryos were injected with *Sox2BD(-)* mRNA (right) or control mRNA (left) in a manner comparable to that for *SoxDBD(-)* mRNA injections. Injection of *Sox2BD(-)* inhibited expression of *Otx2* (B), *Xanf1* (C), *En2* (D), *Krox20* (E), and *HoxB9* (F). Note that inhibition of *HoxB9* expression was detected only in the posterior neural tube (arrowhead) but not in the posterior mesoderm (arrow).



$n=27$). Application of Dex alone did not cause any change in neural differentiation of either the animal caps or the neural plate in vivo (Fig. 6C,D, $n=23$ and 20, respectively). The data above demonstrate that *Sox2*-mediated signaling is required in the ectoderm at or following late gastrula stages, when the neuroectoderm has already expressed early neural genes such as *Zic* genes (see Fig. 5). We next tested whether *Sox2*-mediated signaling is required at later stages. *N-CAM*

expression starts at the end of gastrulation (stage 13 by in situ hybridization). When Dex was applied from stage 14 onwards, *N-CAM* expression decreased in the animal caps (75%, $n=24$; Fig. 6H) and embryos injected with *Sox2BD(-)-GR* (74%, $n=34$; Fig. 6L), suggesting that *Sox2*-class factors play an essential role in the maintenance of late neural markers such as *N-CAM*.

Neural precursors with reduced *Sox2* signaling are not able to become either neural or epidermal cells

Finally, we analyzed the fate of the cells that failed to differentiate into neural tissues due to lack of *Sox2* function. Fig. 7A shows RT-PCR analysis of injected animal caps. Overexpression of *dnBMPR* suppressed the epidermal marker *Keratin* and induced the neural marker *N-CAM* (lane 2, 3). *Sox2BD(-)* significantly suppressed this *N-CAM* induction by *dnBMPR* whereas *Keratin* expression remained undetectable (lane 4). This is not due to inhibitory effects of *Sox2BD(-)* directly on epidermogenesis since *Keratin* expression was intact in the caps that were injected with *Sox2BD(-)* but without *dnBMPR* (lane 5). In addition, *Sox2BD(-)* did not induce the cement gland marker *XAG-1* at the cost of *N-CAM* (lane 4). These data demonstrate that the cells in which neural differentiation has been initiated can neither proceed toward neural fate nor adopt an epidermal fate when *Sox2* function is inhibited. This is consistent with the idea that *Sox2*-class factors are required for a secondary stage of differentiation of

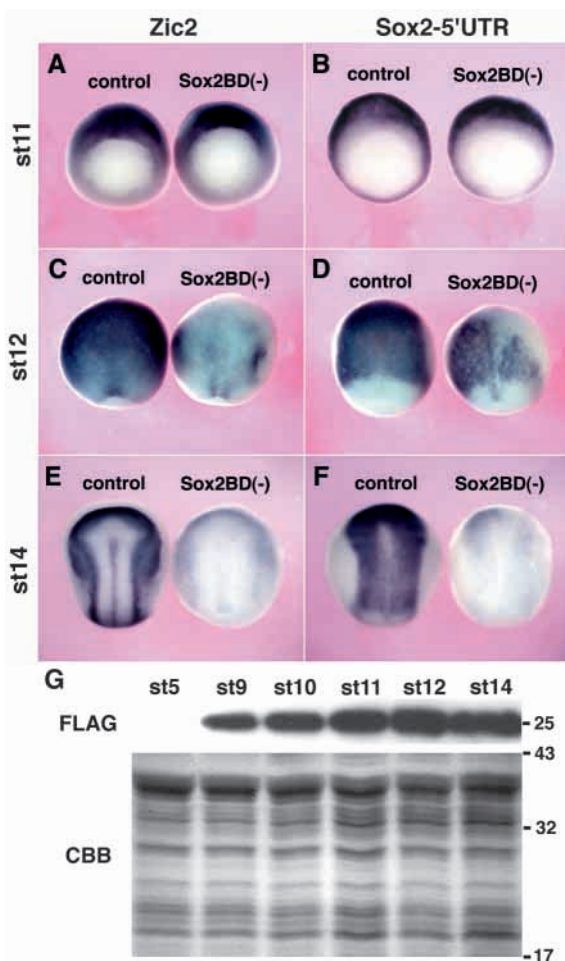


Fig. 5. Effects of *Sox2BD(-)* on early neural marker genes.

(A-F) Embryos were injected with *Sox2BD(-)* mRNA (right) or control mRNA (left) as described for Fig. 4. Whole-mount in situ hybridization was performed with *Zic2* (A,C,E) and *Sox2-5'UTR* (B,D,F) probes. No significant inhibition of *Zic2* or *Sox2* expression was detected at stage 11 (A,B). In embryos at stage 12 and 14, injection of *Sox2BD(-)* reduced expression of both early neural markers (C-F). (G) Accumulation of *Sox2BD(-)* protein (FLAG-tagged) shown by western blot analysis. Embryos injected with FLAG-tagged *Sox2BD(-)* mRNA were harvested at stages indicated above each lane. Western blotting with anti-FLAG antibody (upper panel) gave a single band of the expected size (25 kDa) in each lane. This 25 kDa band was not detected when control *lacZ*-injected embryos were used (not shown). CBB staining of SDS-PAGE (lower panel) is presented as loading controls.

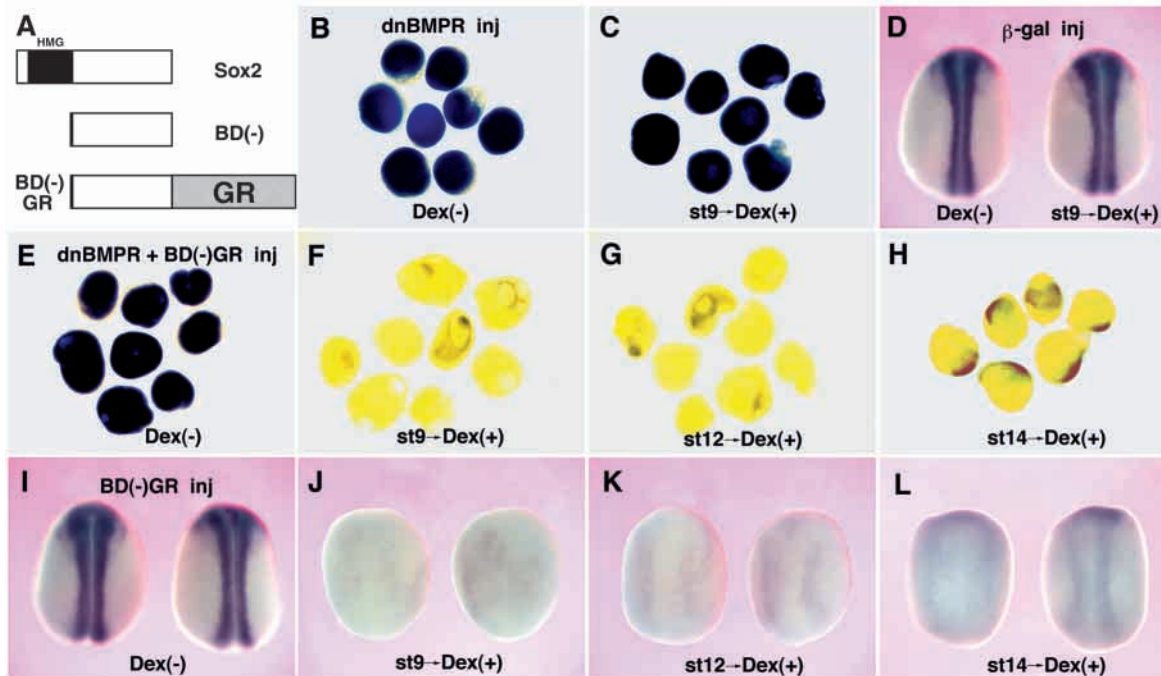


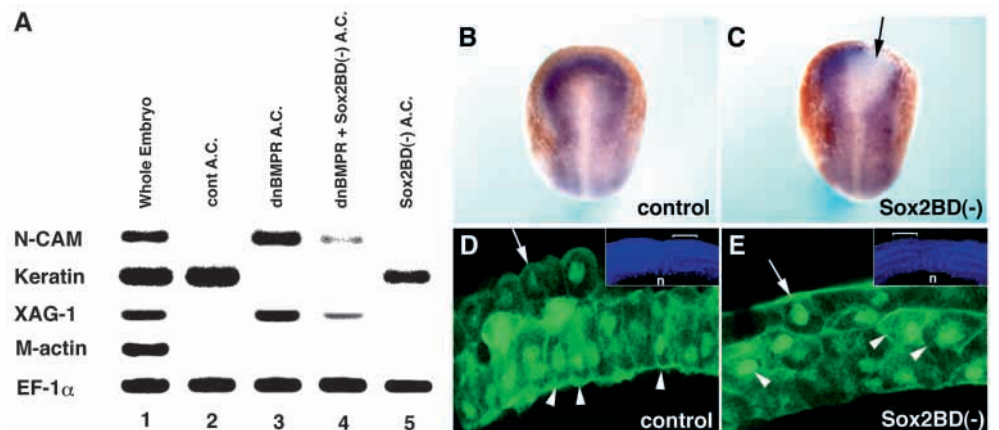
Fig. 6. Temporal requirement of *Sox2*-mediated signaling during neural differentiation. *Sox2BD(-)-GR* was generated by adding the *GR* sequence to the carboxyl terminus of *Sox2BD(-)* (A, bottom). (B) *N-CAM* expression in animal caps neuralized with *dnBMPR*. Application of Dex did not affect *N-CAM* mRNA expression either in the animal caps injected with *dnBMPR* (C) or in the embryo injected with control *LacZ* mRNA (D). In animal cap assays (E-H), neural differentiation triggered by *dnBMPR* was not inhibited by coinjecting *Sox2BD(-)-GR* mRNA in the absence of Dex (E) while the induction of *N-CAM* in the injected caps was significantly suppressed when Dex was added to the culture medium from stage 9 onwards (F), stage 12 (G) or stage 14 onwards (H). (I-L) 200 pg of *Sox2BD(-)-GR* mRNA was injected into each animal blastomere of the 8-cell embryo. Without Dex treatment, injection of *Sox2BD(-)-GR* had little effect on *N-CAM* expression (I). Significant suppression of *N-CAM* was observed when the injected embryos were treated with Dex from stage 9 onwards (J), stage 12 (K) or stage 14 (L) onwards. The extent of suppression of (H) and (L) tended to be less complete than that found in Dex treatment from earlier stages (F,G,J,K).

neuroectoderm rather than for the initial binary decision (neural or epidermal).

This idea was also tested *in vivo*. At the 8-cell stage, 400 pg of *Sox2BD(-)* mRNA was injected into one animal blastomere and the embryo was analyzed with double *in situ* hybridization at stage 15 (mid-neurula). In the control embryo (Fig. 7B), *N-CAM* (purple) and Keratin (brown) signals were detected in

neural and epidermal tissues, respectively, leaving no gap between the two markers. In contrast, the *Sox2BD(-)*-injected embryo (Fig. 7C) had a reduced *N-CAM*-expressing area, and showed the appearance of a *N-CAM*-negative, Keratin-negative region instead (arrow; 93%, $n=40$). The area expressing *XAG-1* did not expand in the embryos injected with *Sox2BD(-)* ($n=25$; not shown). These results indicate that

Fig. 7. *Sox2BD(-)* does not promote epidermogenesis at the cost of neural tissues. (A) RT-PCR analysis of stage 19 animal caps injected with control (lane 2), *dnBMPR* (lane 3), *dnBMPR + Sox2BD(-)* (lane 4) or *Sox2BD(-)* (lane 5) mRNA. Primers specific for *N-CAM*, epidermal Keratin, *XAG-1*, muscle-actin (*M-actin*) and the control *EF-1 α* were used. (B,C) Double-labeled *in situ* hybridization with *N-CAM* (purple) and Keratin (brown) probes. The embryos were injected with control mRNA (B) or *Sox2BD(-)* mRNA (C) at one animal blastomere of the 8-cell embryo. The neural plate region that failed to express *N-CAM* (arrow) did not give Keratin signals, either. (D,E) Confocal microscopy images of the neural plate region of neurula embryos. Green fluorescence shows the progeny of the blastomere that was injected with control *lacZ* and *EGFP* mRNA (D) or *Sox2BD(-)* and *EGFP* mRNA (E). The insets are low magnification images showing the nuclear DAPI staining. The brackets indicate the areas shown in high magnification GFP images. n, notochord; arrows, cuboidal cells in the superficial layer; arrowheads, cells in the sensorial layer.



Sox2BD(-) inhibits differentiation of neuroectoderm cells without re-directing the cells into epidermal fate.

Finally, we examined morphological changes of the ectodermal cells injected with *dnSox2* mRNA. One animal blastomere of the 8-cell embryo was injected with tracer *GFP* mRNA together with either *Sox2BD(-)* or control mRNA. The embryos were fixed at the mid-neurula stage, sectioned and analyzed with confocal microscopy. The cells derived from the blastomere injected with control and *GFP* mRNA formed two-layered neuroectoderm characteristic of *Xenopus* embryos (Fig. 7D; inset, nuclear staining at a lower magnification). The superficial layer was composed of short cuboidal cells (arrow) and the deep (sensorial) layer contained tall columnar neuroepithelial cells (arrowheads). In contrast, the *Sox2BD(-)*-injected cells in the neural plate region did not show either columnar or squamous appearance in the sensorial layer (arrows in Fig. 7E; $n=10$). This is in good agreement with the data above shown with *N-CAM* and *Keratin* markers. Later during the tailbud stages, *Sox2BD(-)*-injected ectoderm started to peel off from the embryo (100%, $n=35$; not shown). We tested by TUNEL assay (Hensey and Gautier, 1997) whether the injected cells went through massive apoptosis. Both at late neurula stages and at tailbud stages, only a small number of embryos showed elevated TUNEL signals in the injected regions (2.6%, $n=75$; not shown), suggesting that improper differentiation changed adhesion characteristics of the cells during tailbud stages without causing programmed cell death.

DISCUSSION

Inhibition of *Sox2*-mediated function by dominant-negative *Sox2* constructs

The Sox family factors including Sox2 are known to require cofactors (or partners) for their transactivation activities (Kamachi et al., 1995). The HMG domain is responsible for DNA-binding and the rest of the protein is required for interaction with cofactors and for transactivation (Kamachi et al., 1999). Using distinct regions of *Sox2*, we generated two different mutant *Sox2* constructs that have dominant-inhibitory effects over wild-type *Sox2*. One contained the *Sox2* DNA-binding domain and was fused with the *en* repressor region. This is now a frequently used method of making a dominant-negative factor in *Xenopus* studies, and it is believed that the dominant-negative chimera works by binding to target genes (Conlon et al., 1996). The other construct that we used was a *Sox2* cDNA lacking most of the DNA-binding domain. A similar construct was successfully used in the case of *SoxD* (Mizuseki et al., 1998b). Since it cannot bind to the target DNA, it is likely to act by competing with endogenous Sox2 and Sox2-like factors for the essential cofactors.

Both constructs showed an efficient dominant-inhibitory effect on neuralizing activity of *Sox2* in the FGF-treated animal caps (Fig. 1). Rescue experiments demonstrated that the inhibition was not due to secondary toxic effects. This allowed us to test if *Sox2* is required for neural differentiation, which is known to involve the blockade of BMP signaling.

Requirement of *Sox2*-mediated signaling in neural differentiation of *Xenopus* ectoderm

The dominant-negative experiments showed that signaling

mediated by *Sox2* is required for *Xenopus* ectoderm to express neural markers such as *N-CAM*. In animal caps injected with *dnBMPR*, neural differentiation was inhibited by coinjection of *dnSox2* mRNAs. The conclusion was strengthened by the observation that two constructs containing distinct *Sox2* regions (*Sox2BD(-)* and *Sox2-EnR*) gave similar results.

In vivo studies showed that *Sox2*-mediated signaling is essential for all the neural markers studied at neurula stages. *Sox2BD(-)* prevented the dorsal ectoderm from expressing the pan-neural marker *N-CAM*, the neuronal markers *N-tubulin* and *neurogenin*, and the neural crest markers *Slug* and *fhx6* (Fig. 3). Thus, both CNS and neural crest formations require *Sox2* signaling by the time of neurula stages. This effect was unlikely to be mediated secondarily by suppressing development of dorsal mesoderm (which can induce neural tissues) since the *Chd* and *MyoD* markers were intact (Fig. 3D,E).

Differential requirement for *Sox2* and *SoxD* signals in early neural development

In our previous reports, we identified two distinct classes of *Sox* genes expressed in the frog gastrula ectoderm. *Sox2* is expressed in the presumptive neural ectoderm from late blastula stages onwards, while *SoxD* expression is first pan-ectodermal and becomes neural-specific during mid-gastrula stages. In structure, *SoxD* is distantly related to other Sox factors including *Sox2*. Beside the conserved HMG DNA-binding domain, *SoxD* shows no homology to any *Sox* genes. Overexpression of *SoxD* induces neural differentiation of ectoderm while that of *Sox2* per se is not sufficient to initiate neural differentiation in animal cap ectoderm.

In animal cap assays, injection of *dnSoxD* and that of *dnSox2* have similar effects. Both inhibit expression of the pan-neural marker *N-CAM* induced by *dnBMPR*. However, in vivo studies show different outcomes. Injection of *dnSoxD* suppresses development of the anterior neural tissues, mostly forebrain, whereas overexpression of *Sox2BD(-)* prevents differentiation of neural tissues in general, both in the anterior and posterior regions. This demonstrates that *Sox2* and *SoxD* have distinct roles in neural development.

Possible timing of requirement of *Sox2* in *Xenopus* neuroectoderm

By using an inducible *Sox2BD(-)* construct fused to *GR*, we attempted to analyze temporal requirement of *Sox2* signaling for neural differentiation. This construct showed inhibitory effects on neural differentiation of ectoderm in vivo and in the animal cap, in a Dex-dependent manner. The common understanding concerning the conditional regulation of GR-fused nuclear factors is that the GR domain is tethered to the cytoplasmic protein HSP90 and is released upon binding to a GR ligand (reviewed in Smith and Toft, 1993). As discussed above, *Sox2BD(-)* is likely to act by competing for cofactors with endogenous *Sox2* and its related proteins. Therefore, nuclear localization of *Sox2BD(-)* protein may be essential for its dominant-negative effects. This explains how *Sox2BD(-)-GR* activity is dependent on Dex. A similar usage of *GR* to make a conditional dominant-negative construct was recently reported (Horb and Thomsen, 1999).

Both the animal cap experiments and in vivo studies showed that inhibition of neural differentiation was seen by 'activation'

of *dnSox2* not only from the late blastula stage but also from the late gastrula stage onwards. In amphibia (newt), classical experiments have shown that neural fate of dorsal ectoderm is determined by the time of late gastrula stages (Spemann, 1918). Although detailed studies have not yet been reported in *Xenopus* as to when the neuroectoderm becomes irreversibly neural, columnarization of neuroectoderm cells is seen by histology within the sensorial layer during late gastrulation (stage 12-13; Hausen and Ribesell, 1991). These observations strongly suggest that the dorsal ectoderm of late gastrula has received and responded to inductive neuralizing signals. In our study (Fig. 6), late gastrula neuroectoderm could not further differentiate into neural tissues when *Sox2* signaling was inhibited, suggesting that *Sox2*-class factors are essential for presumptive neural ectoderm to further differentiate beyond gastrula stages.

In this study, we demonstrated that *Sox2* signaling is required during secondary stages of neural differentiation starting from late gastrula. However, it remains unclear whether *Sox2* function is necessary for the initial step of neural induction which occurs around stage 10. One possible experiment is to analyze effects of inducible *Sox2BD(-)* by adding Dex at stage 9 and removing it by stage 12. However, this experiment proves difficult since hydrophobic Dex can be deposited efficiently in the yolk lipid, making the Dex removal incomplete. Instead, we examined effects of *Sox2BD(-)* mRNA injection on expression of *Zic* and *Sox2* transcripts at the early gastrula stage (Fig. 5). In this case, *Sox2BD(-)* did not suppress expression of these two neural markers at stage 11, suggesting that inhibition of *Sox2* signaling has little effects on initial steps of neural induction.

Permissive role of *Sox2* in neural development

As described previously, overexpression of *Sox2* mRNA per se has little effects on animal cap ectoderm. When combined with FGF, *Sox2* can modify the responsiveness of animal cap cells to FGF neuralizing signals (Mizuseki et al., 1998a; Fig. 1). *Sox2* alone is not sufficient to direct cells to neural fate but rather plays a role in changing the competence of the ectoderm. When 100 pg of *Sox2* mRNA was injected into all the animal cells of 8-cell embryos, overexpression of *Sox2* had very weak effects, if any, on the expression of neural markers at neurula stages (our unpublished observations). Higher doses of *Sox2* mRNA caused non-specific effects such as exogastrulation. Therefore, as of yet, we have no particular evidence for instructive roles of *Sox2* in early neural development.

In this study, we reported data that support permissive roles of *Sox2* in developing nervous systems. *Sox2* and/or its close relatives are necessary for the ectoderm to develop into neural tissues during secondary steps of neural differentiation. However, it remains to be known exactly which members of the *Sox2*-subfamily are responsible for this role. Being so closely related in structure and in expression pattern, it is likely that *Sox2*-subfamily genes have largely redundant functions. Additionally, it also remains to be clarified precisely which signaling steps in the downstream cascade of neural induction require the presence of *Sox2*-class factors. To study detailed gene interaction between early regulators of neural differentiation, analysis of the promoters of early neural marker genes by using the transgenic frog technique may prove useful (Kroll and Amaya, 1998). This new technique should provide

us with information about the mode of action of *Sox2* on neural-specific transcription in future.

In closing, our present results have provided the first solid evidence for an essential role of *Sox2*-class genes in the forming neuroectoderm. Further studies on the role of *Sox2* should help us dissect the regulatory cascade of neural development into individual steps.

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