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Pax6 regulates specification of ventral neurone subtypes in the hindbrain by establishing progenitor domains

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SUMMARY

Recent studies have shown that generation of different kinds of neurones is controlled by combinatorial actions of homeodomain (HD) proteins expressed in the neuronal progenitors. Pax6 is a HD protein that has previously been shown to be involved in the differentiation of the hindbrain somatic (SM) motoneurones and V1 interneurones in the hindbrain and/or spinal cord. To investigate in greater depth the role of Pax6 in generation of the ventral neurones, we first examined the expression patterns of HD protein genes and subtype-specific neuronal markers in the hindbrain of the *Pax6* homozygous mutant rat. We found that *Islet2* (SM neurone marker) and *En1* (V1 interneurone marker) were transiently expressed in a small number of cells, indicating that *Pax6* is not directly required for specification of these neurones. We also observed that domains of all other HD protein genes (*Nkx2.2*, *Nkx6.1*, *Irx3*, *Dbx2* and *Dbx1*) were shifted and their boundaries became blurred. Thus, *Pax6* is required for establishment of the progenitor domains of the ventral neurones. Next, we performed *Pax6* overexpression experiments by electroporating rat embryos in whole embryo culture. *Pax6* overexpression in the wild type decreased expression of *Nkx2.2*, but ectopically increased expression of *Irx3*, *Dbx1* and *Dbx2*. Moreover, electroporation of *Pax6* into the *Pax6* mutant hindbrain rescued the development of *Islet2*-positive and *En1*-positive neurones. To know reasons for perturbed progenitor domain formation in *Pax6* mutant, we examined expression patterns of Shh signalling molecules and states of cell death and cell proliferation. Shh was similarly expressed in the floor plate of the mutant hindbrain, while the expressions of *Ptc1*, *Gli1* and *Gli2* were altered only in the progenitor domains for the motoneurones. The position and number of TUNEL-positive cells were unchanged in the *Pax6* mutant. Although the proportion of cells that were BrdU-positive slightly increased in the mutant, there was no relationship with specific progenitor domains. Taken together, we conclude that *Pax6* regulates specification of the ventral neurone subtypes by establishing the correct progenitor domains.

Key words: Neuronal specification, Progenitor domains, Hindbrain, *Pax6*, *Small eye* rat, Somatic motoneurones, Interneurones, Homeodomain proteins

INTRODUCTION

In the developing central nervous system (CNS) of the vertebrate, various types of neurone are produced in precise positions along the dorsoventral (DV) and anteroposterior (AP) axes of the neural tube. Recent studies have drawn a scenario that gradient signals from the ventral and dorsal midline structures pattern the neural plate and neural tube (reviewed by Jessell, 2000). The diffusion of a secreted signalling factor Sonic hedgehog (Shh) from the notochord and the floor plate has been proposed to establish a ventral-to-dorsal gradient of Shh activity within the neural tube that directs subsequent patterns of neurogenesis (Roelink et al., 1994; Chiang et al., 1996; Ericson et al., 1996; Teleman et al., 2001). By contrast, several secreted molecules that belong to bone morphogenetic protein (BMP) family regulate the patterning of dorsal cell types (Liem et al., 1997; Lee and Jessell, 1999).

The role of Shh in the patterning of ventral neural tube has been well studied in the spinal cord. Several homeodomain (HD) proteins are expressed in specific domains of the ventricular zone of the developing spinal cord (Goulding et al., 1993; Ericson et al., 1997; Pierani et al., 1999). They are categorised into two classes: the genes whose expression is repressed by Shh (Pax6, Dbx2, Irx3, Dbx1 and Pax7) are known as class I, while those whose expression is induced by Shh (Nkx2.2 and Nkx6.1) are class II (Briscoe et al., 2000). Misexpression and loss of functions of the HD protein genes can cause alteration of the neuronal subtypes (Briscoe et al., 1999; Briscoe et al., 2000; Sander et al., 2000; Pierani et al., 2001). These data suggest that the progenitor cell identity and the neuronal subtypes are regulated via distinct regionalization of the ventricular zone in the neural tube represented by the combinatorial expression of the HD proteins, i.e., the HD code.

Members of Pax family proteins are HD-containing transcription factors, and Pax6 is the most characterised member (reviewed by Gruss and Walther, 1992; Hill and Hanson, 1992; van Heyningen, 1998; Gehring and Ikeo, 1999). During development, *Pax6* is expressed in the dorsal forebrain, including a region that gives rise to the cortex, dorsal thalamus and pretectum, and functions in patterning the brain (Walther and Gruss, 1991; Stoykova et al., 1997; Pratt et al., 2000; Osumi, 2001). In the hindbrain and spinal cord, *Pax6* is expressed in the ventral region and plays crucial roles in generation of ventral neurones. Five types of neurones that are respectively marked with expressions of specific transcriptional factors differentiate in the ventral hindbrain. They are, from ventral to dorsal, branchiomotor (BM) and somatic (SM) motoneurones, and V2, V1 and V0 interneurones (Fig. 1A). In the hindbrain of *Pax6* homozygous mutant mice and rats, the SM neurones and V1 interneurones are missing, while the BM neurones increased in number (Osumi et al., 1997; Ericson et al., 1997; Burill et al., 1997; Osumi and Nakafuku, 1998; Sun et al., 1998). However, in the *Pax6* mutant spinal cord, SM neurones do develop and a small number of V1 interneurones appear at later stages. Therefore, how Pax6 functions in ventral neurone development is still enigmatic.

In the present study, we first performed detailed time-course analyses on expression of neuronal subtype markers in the hindbrain of the *Pax6* homozygous rat embryo. We found that *Islet2* and HB9/MNR2 (SM neurone markers) and *En1* (V1 interneurone marker) were transiently expressed in a small number of cells, indicating that *Pax6* expression is not an absolute requirement for the differentiation of these neurones. By comparing the expression of HD protein genes in the wild type and the mutant, we found that the domains of all other HD protein genes (i.e. *Nkx2.2*, *Nkx6.1*, *Irx3*, *Dbx2* and *Dbx1*) were shifted dorsoventrally and their boundaries became fuzzy. Thus, *Pax6* seems to be required for establishment of the progenitor domains for the ventral neurones. Moreover, *Pax6* overexpression in the wild type altered the expressions of other HD protein genes, and *Pax6* electroporation into the *Pax6* mutant hindbrain rescued the expression of *Islet2* and *En1*, but only in the normal positions. To know reasons for perturbed progenitor domain formation in *Pax6* mutant, we further examined patterns of Shh signalling and cell kinetics. The expression of *Ptc1*, *Gli1* and *Gli2* was slightly altered in the mutant, although Shh itself was similarly expressed in the floor plate, suggesting that *Pax6* may modify the Shh signalling in the progenitor domains for the motoneurones. The position and number of TUNEL-positive cells were unchanged in the *Pax6* mutant. Although the proportion of cells that were BrdU-positive increased in the mutant, there was no relationship with progenitor domains. These findings suggest that *Pax6* regulates the specification of the ventral neurones by establishing the correct progenitor domains.

MATERIALS AND METHODS

Animals

Pax6 homozygous rat embryos were obtained by inter-crossing male and female heterozygotes of *Small eye* rats (*rSey*^{2}) (Osumi et al., 1997). Wild-type Sprague-Dawley (SD) rats were purchased from Charles River Japan. The midday of the vaginal plug was designated as 0.5 day. E10.5-13.5 embryos were used for analyses. In this study, 24- and 36-somite stages were defined as E11.5 and E12.5, respectively. The following experimental procedures were approved by The Committee for Animal Experiment of Tohoku University Graduate School of Medicine.

Immunohistochemistry

Immunohistochemistry on frozen sections was performed as described previously (Osumi et al., 1997). Anti-Pax6 rabbit polyclonal antibody (Inoue et al., 2000) was used at 1:1000 dilution. 40.2D6 anti-Islet1/2 (1:100) (Ericson et al., 1992), 74.5A5 anti-Nkx2.2 (1:50) (Ericson et al., 1997) and 81.5C10 anti-MNR2 (1:25) (Tanabe et al., 1998) mouse monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa), and used at the specified dilution. Although rat HB9 homologue has not been identified, the staining pattern with the anti-chick MNR2 monoclonal antibody was similar to previous reports for HB9 expression in the mouse embryo (Arber et al., 1999; Thaler et al., 1999). As amino acid sequence of MNR2 is very similar to that of HB9, it is possible that anti-chick MNR2 antibody recognised HB9 and/or HB9 related protein on the rat tissue. Anti-Lim3 rabbit polyclonal antibody was kindly provided by S. L. Pfaff and used at 1:5000 (Sharma et al., 1998). Anti-GFP mouse monoclonal antibody was purchased from Clontech and used at 1:1,000. Antigen enhancement was performed according to the method described previously (Osumi et al., 1997). As secondary antibodies, Cy3-conjugated affinity-purified donkey anti-rabbit IgG (1:600) and anti-mouse IgG (1:400) solutions, FITC-conjugated affinity-purified donkey anti-mouse IgG (1:200) and anti-rabbit IgG (1:200) solutions, and biotin-conjugated affinity-purified donkey anti-rabbit IgG (1:200) and anti-mouse IgG (1:200) solutions (Jackson Immunoresearch Laboratories, Chemicon International, respectively) were used. ABC kit (Vector Laboratories) and Metal enhanced DAB kit (Pierce) were used for detection with horseradish peroxidase.

In situ hybridisation

RT-PCR was performed to obtain rat cDNA clones for templates. Total RNA taken from the head of E13.5 SD rat embryos was purified by RNeasy column (Qiagen) and cDNA was synthesised using reverse transcriptase and oligo dT primer (Superscript preamplification system; Gibco BRL). Oligonucleotides used to amplify cDNAs were as follows: *Chx10*, 5′-AGCGCTGAGCAAGCCAAAT-3′ and 5′-CTAAGCCATGTCCTCCAGCT-3′; *Dbx1*, 5′-TCTAGAATGATGTT-CCCCGG-3′ and 5′-CTAGGACACCGTGATTTCCT-3′, according to previously described mouse sequences (Liu et al., 1994; Lu et al., 1994). Amplification was performed with a thermal cycler (Mastercycler Gradient; Eppendolf) using *Taq* DNA polymerase (Promega) using the following protocol: denaturation for 5 minutes at 96°C, annealing for 1 minute at 62.4°C (*Chx10*) or 63.4°C (*Dbx1*), extension for 1 minute at 72°C, 35 cycles. These cDNA fragments, including the open reading frames, were cloned into pBluescript II SK (–) (Stratagene) and sequenced to confirm they were rat counterparts. Rat *En1*, *Nkx2.2*, *Shh* and *Pax6* cDNAs were used previously (Matsuo et al., 1993; Osumi and Nakafuku, 1998), and rat *Islet2* cDNA was a kind gift from S. Pfaff (Tsuchida et al., 1994). Rat *Smoothened* cDNA (Stone et al., 1996) was kindly provided by A. Rosenthal. To synthesise other probes, mouse cDNA clones were used. *Evx1* cDNA (Bastian and Gruss, 1990) was provided by M. Goulding, *Nkx6.1* cDNA (Qiu et al., 1998) by J. Rubenstein, *Irx3* cDNA (Bosse et al., 1997) by P. Gruss, *Dbx2* cDNA (Shoji et al., 1996) by N. Takahashi, *Gli1* and *Gli2* cDNAs (Ding et al., 1999) by H. Sasaki, and *Patched1* cDNA (Goodrich et al., 1996) by M. Scott. Digoxigenin-labelled antisense riboprobes were generated with T3 or T7 RNA polymerase (Promega). In situ hybridisation on frozen sections was performed as described previously (Ishii et al., 2000). In some cases,

immunohistochemistry was performed on the same sections after in situ hybridisation.

Electroporation into cultured rat embryos

The method used for electroporation into cultured mammalian embryos was described previously (Osumi and Inoue, 2001). Chamber-type electrodes (8×20 mm electrodes and 20 mm distance between electrodes) were used in this study. To construct *Pax6* expression plasmid, blunted *Spe*I-*Kpn*I fragment of mouse *Pax6* cDNA (a kind gift from P. Gruss) (Walther and Gruss, 1991) was inserted into blunted *Hin*dIII-*Kpn*I site of *pCAX* expression plasmid containing the cytomegalovirus enhancer and chicken β-actin promoter (*pCAX* and *pCAX-GFP* plasmids were kindly provided by the late K. Umesono). At E10.75 stage, the uterus was dissected out from anaesthetised *rSey*^{2} heterozygous females, and littermate embryos were dissected out with their placenta and yolk sac intact. After a 2 hour preculture, the embryos were transferred into the chamber-type electrodes and DNA solution of *pCAX-mPax6* and *pCAX-GFP* (9:1) dissolved in PBS at 5 mg/ml was injected into the hindbrain. Immediately, square pulses (50 mseconds, 70 V, five times) were sent using an electroporator (CUY21; NEPPA GENE), and the embryos were further cultured. At this point, we could not distinguish homozygous embryos from external features. Twelve hours later when the cultured embryos developed to the stage corresponding to E11.5, the yolk sac was opened and homozygous embryos were identified based on morphological defects in the brain and eyes. In electroporation of the *Pax6* mutant at early E11.5 (22-somite stage), littermate embryos were dissected with the yolk sac opened, and homozygous embryos that were identified from the external features were used for electroporation. Both the wild-type and homozygous embryos were precultured for 2 hours, and electroporated with square pulses (50 mseconds, 70-90 V, five times). The cultured embryos were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C. Selected embryos in which GFP fluorescence was seen only in one side of the neural tube were processed for further analyses as described above. In total, 10 wild-type and eight homozygous mutant embryos at early E11.5, and three mutant embryos at E10.75 were used for analysis.

Assay for cell death and cell proliferation

Cell death was assayed quantitatively by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labelling (TUNEL) as described previously (Wakamatsu et al., 1998) with minor modification. The embryos were fixed with 4% PFA in PBS overnight at 4°C. Frozen sections were treated with 1 mg/ml proteinase K/PBS for 5 minutes at 37°C, and refixed with 4% PFA in PBS for 10 minutes. Sections were incubated with TdT buffer containing boitin-14-ATP (Gibco BRL) and terminal transferase (Roche Molecular Systems) for 1 hour at 37°C. Labelled cells were detected with avidin-Cy3 (1:200, Jackson).

Cell proliferation was assayed quantitatively by pulse-labelling with bromodeoxyuridine (BrdU) of cultured mammalian embryos (Ishii et al., 2000) with minor modification. The E12.5 wild-type and homozygous embryos were precultured for 1 hour, followed by the addition of BrdU solution to the culture medium. The embryos were exposed to BrdU for just 20 minutes and then fixed immediately. Detection of BrdU was performed according to Marusich et al. (Marusich et al., 1994) using anti-BrdU antibody (1:50; Becton Dickinson) and Cy3-conjugated affinity-purified donkey anti-mouse IgG (1:400, Jackson) antibody. Sections were counterstained with DAPI.

In situ hybridisation for *Dbx1* probe was performed on adjacent sections to determine the area for cell count. For count of BrdU-, and TUNEL-positive cells, 10 sections at the r7 level from three *Pax6* homozygous and three wild-type rat embryos were analysed.

RESULTS

V1 interneurones and SM neurones transiently emerge in the hindbrain of the *Pax6* mutant

Previous studies have shown the lack of V1 interneurones expressing *En1* in both the hindbrain and spinal cord in *Pax6* homozygous mutants at E10.5 in mice and at E12.5 in rats (Ericson et al., 1997; Burill et al., 1997; Osumi and Nakafuku, 1998). However, *En1* transcripts are detected in the spinal cord of *Pax6* homozygous mice at E12.5 (Burill et al., 1997). Therefore, we carefully re-explored whether *En* transcripts were detected in the *Pax6* homozygous rat hindbrain from E10.5 to E13.5. In the wild type, *En1*-positive cells were firstly observed at E12.0, and more *En1*-positive cells were observed at E13.0 (Fig. 1E). In the *Pax6* homozygous embryos at E12.0-12.75, *En1* expression was undetectable throughout the hindbrain and spinal cord, but at E13.0 a few *En1*-positive cells were found at r3-6 level of the *Pax6* homozygous embryos (Fig. 1I). At r7 level, transient expression of *En1* was not seen throughout stages analysed (Fig. 1B). At E13.5, *En1* expression at r3-6 diminished in the mutant (Fig. 1B). These results indicate that differentiation of V1 interneurones is not entirely dependent on *Pax6* function in the hindbrain.

Next, we investigated differentiation of other interneurones, including V2 (*Chx10*-positive) and V0 (*Evx1*-positive) populations, in the *Pax6* homozygous embryos. *Chx10* transcripts were detected from E12.0 in the hindbrain of wild-type and *Pax6* homozygous embryos (Fig. 1A,B,D,H). It has previously been reported that cells expressing Chx10 protein are reduced at r7 in the mouse *Pax6* homozygous embryo (Ericson et al., 1997). In the rat *Pax6* homozygous embryo, however, we did observe *Chx10*-positive cells at E12.0-13.5, and the domain of *Chx10* expression expanded somewhat dorsally (bracket in Fig. 1H). The onset of *Evx1* expression was later than that of *En1* and *Chx10*; *Evx1*-positive cells emerged from E12.5 and afterwards in the hindbrain of the wild type (Fig. 1A,F). In the *Pax6* mutant, *Evx1*-positive cells were not observed until E13.5 at r7 level, while they were found to expand somewhat ventrally at r3-6 levels on E12.75-E13.0 (Fig. 1B,J). Thus, differentiation of the ventral interneurones was impaired in the *Pax6* mutant hindbrain.

We have previously demonstrated that mutation in *Pax6* gene results in loss of SM and increase of BM neurones in the rat hindbrain (Osumi et al., 1997). The same defect is seen in the homozygote of small eye mouse mutant (Ericson et al., 1997; Burrill et al., 1997). However, the SM neurone defect is less severe at the cervical level of the spinal cord. Thus, we re-examined the expression of *Islet2* with special reference to embryonic stages and AP levels. At r5 and r7 levels in the wild-type rat hindbrain, *Islet2* expression was first detected at E11.5 and maintained at E13.5 (Fig. 1A). In the *Pax6* homozygous embryos, *Islet2* transcripts were not detected until E12.5, as reported previously (Fig. 1B) (Osumi et al., 1997). However, we found a few cells expressing *Islet2* at r7 level during E12.75-13.0 in the *Pax6* mutant embryos (Fig. 1Q). We further checked whether the expression of HB9/MNR2, another marker for SM neurones, transiently emerged in the *Pax6* homozygous embryos. In the wild-type rat embryo, HB9/MNR2 immunoreactivity was observed from E11.5 (Fig. 1A). In the *Pax6* mutant, cells expressing HB9/MNR2 were not seen in the E12.5 mutant hindbrain, but a few cells expressing

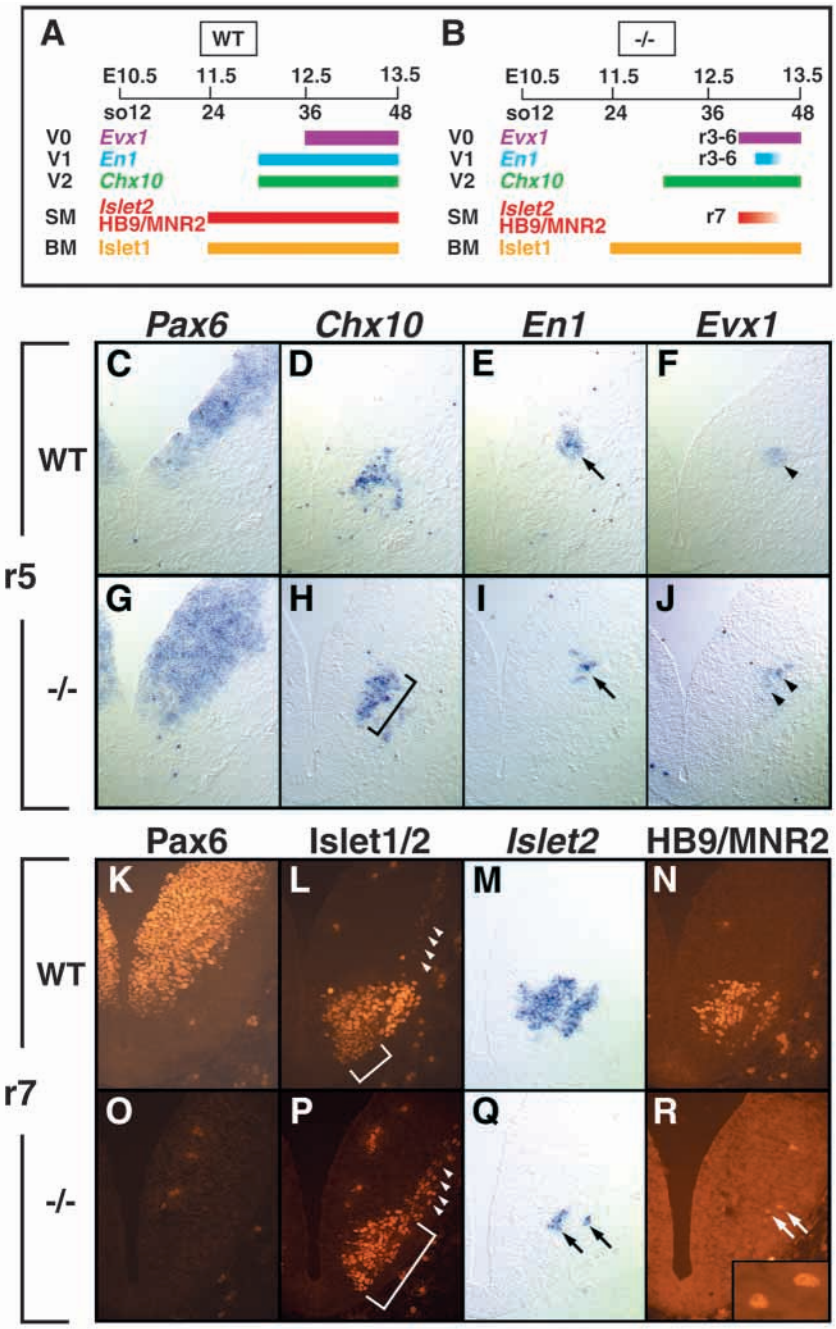

Fig. 1. Mis-specification of ventral neurones in the *Pax6* mutant hindbrain. (A,B) Summary of detailed time-course analyses on expression of ventral neurone markers in the wild-type (A) and *Pax6* homozygous (B) embryos. Five types of neurones develop in the ventral hindbrain: from ventral to dorsal, branchiomotor (BM) and somatic (SM) motoneurones, and V2, V1 and V0 interneurones, with distinct marker expressions. *Pax6* is already expressed in the hindbrain at E10.5 (not shown here). During normal development, SM neurones expressing *Islet2* and HB9/MNR2 emerge by E11.5, and expression of HB9/MNR2 and *Islet2* is maintained at E13.5. In the *Pax6* mutant, a small number of cells expressing *Islet2* and HB9/MNR2 are transiently seen during E12.75-13.0 at r7 level. In the wild type, *En1* expression is first detected at E12.0. In the *Pax6* mutant, *En1* is transiently expressed in a small number of cells at r3-6 levels of E13.0-13.25 embryo. (C-J) Expression patterns of ventral interneurone markers. Adjacent sections of E13.0 the wild-type (C-F) and *Pax6* mutant (G-J) hindbrain at r5 level hybridised with *Pax6* (C,G), *Chx10* (D,H), *En1* (E,I) and *Evx1* (F,J) antisense probes. (C,G) *Pax6* is expressed in the ventricular zone of the ventrolateral domain in both the wild-type and mutant neural tubes. (D,H) In the *Pax6* mutant, *Chx10*-positive cells are located in a slightly wider region than those seen in the wild type, spreading more dorsally (see bracket in H). (E,I) In the wild type, *En1*-positive cells are detected in the region dorsal to *Chx10* expression (arrow). (I) In the *Pax6* mutant, *En1*-positive cells are undetectable at E12.5 (not shown here) (Ericson et al., 1997), a small number of *En1*-positive cells are observed in a similar dorsoventral position (arrow). (F,J) In the wild type, *Evx1*-positive cells are detected in the dorsally adjacent region (arrowhead in F). In the mutant, *Evx1* expression seems to expand ventrally at r5 (arrowheads in J). (K-R) Expression of motoneurone markers in the hindbrain. Adjacent sections of E12.75 wild-type (K-N) and *Pax6* mutant (O-R) hindbrain at r7 level. Distribution of Pax6 (K,O), Islet1/2 (L,P) and HB9/MNR2 (N,R) proteins, and *Islet2* transcripts (M,Q). (K,O) In the wild type, Pax6 is observed in the ventricular zone of the ventrolateral domain of the neural tube (K). In the *Pax6* mutant, immunoreaction for Pax6 is not detected with the antibody that recognises the C-terminal region of normal Pax6 protein (O). (L-N) During normal development, SM neurones are positive for Islet1, *Islet2* and HB9/MNR2, while BM neurones (bracket in L) are negative for *Islet2* and HB9/MNR2. Some of BM neurones are located dorsolaterally, as they migrate dorsally at this stage (arrowheads). (P-R) In the *Pax6* mutant, *Islet2*- and HB9/MNR2-positive cells are missing at E12.5 (not shown here) (Osumi et al., 1997), but a small number of *Islet2*-positive and MNR2/HB9-positive cells are observed at E12.75 (arrows in Q,R). Inset in (R) shows higher magnification of HB9/MNR2-positive cells indicated by white arrows.

HB9/MNR2 were observed at r7 level of E12.75-13.0 (Fig. 1R). Such *Islet2* and HB9/MNR2-positive cells were no longer detected at E13.5 in the mutant (Fig. 1B). As for another marker of SM neurones, Lim3, it was expressed in most of *Islet2*-positive cells of E11.5-13.0 wild-type hindbrain (data not shown) (Varela-Echavarría et al., 1996). However, *Islet2*-positive cells transiently observed in E12.75-13.0 mutant did not express Lim3 (data not shown).

In summary, emergence of SM neurones and V1 interneurones in the hindbrain, although transiently, suggests that *Pax6* is not directly required for induction of these cell types.

Progenitor domain formation is perturbed in the *Pax6* mutant

Transient emergence of small number of SM neurones and V1 interneurones prompted us to assume that neuronal progenitor domains for these populations become narrower in the *Pax6*

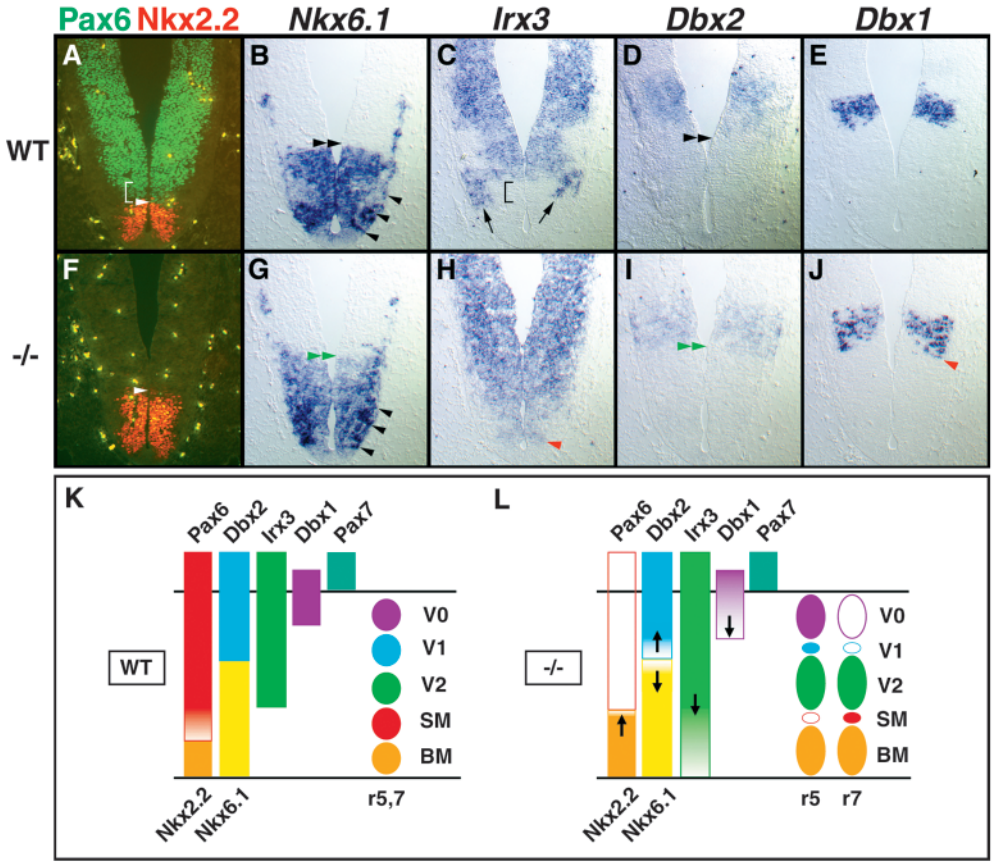

Fig. 2. Expression patterns of homeodomain (HD) protein genes in the hindbrain. (A-J) Adjacent sections of E12.5 wild-type (A-E) and *Pax6* mutant (F-J) hindbrains at r7 level. Double immunostaining for Nkx2.2 and Pax6 (A,F), and in situ hybridisation for *Nkx6.1* (B,G), *Irx3* (C,H), *Dbx2* (D,I), and *Dbx1* (E,J). (A,F) In the wild type, expression of Nkx2.2 is observed in the ventricular zone, and the expression domain is adjacent ventrally to the floor plate and dorsal to Pax6 domain (white arrowhead in A). In the *Pax6* homozygous embryo, Nkx2.2 domain expands dorsally (white arrowhead in F). In the wild type, Pax6 protein expression is at a very low level in most of the ventral area (white bracket in A) from which SM neurones develop. Immunoreactivity for Pax6 is not detected in the *Pax6* mutant (F). (B,G) In the wild type, *Nkx6.1* is localised in the ventral domain excluding the floor plate and adjacent to the *Dbx2* domain (double black arrowhead). In the *Pax6* mutant, the dorsal boundary of *Nkx6.1* expression slightly became blurred (double green arrowhead). *Nkx6.1* expression is also seen in BM neurones in the mantle zone of both the wild type and *Pax6* mutant (arrowheads in B,G). (C,H) In the wild type, *Irx3* is expressed in the regions that overlap with Pax6, except the SM neurone precursor domain (bracket in C). In the *Pax6* mutant, *Irx3* domain expands ventrally into Nkx2.2 domain (red arrowhead). In the wild type, *Irx3* transcripts are also detected in SM neurones (arrows in C), while such population is not seen in the *Pax6* mutant (H). (D,I) In the wild type, the expression domain of *Dbx2* is ventral to the *Nkx6.1* domain (black double arrowhead), and also covers a more dorsal region than *Dbx1* domain. In the *Pax6* mutant, The ventral boundary of *Dbx2* expression also becomes blurred (green double arrowhead in I). (E,J) In the wild type, expression of *Dbx1* is confined to a narrow region within the ventrolateral neural tube. In the *Pax6* mutant, *Dbx1*-positive cells decrease in number and expand ventrally (red arrowhead in J). (K,L) Illustration of the expression of HD protein genes and subtypes of ventral neurones in the hindbrain of the wild type (K) and *Pax6* mutant (L). In the *Pax6* mutant rat, expression domains of HD protein genes are shifted, the boundaries become blurred, and ventral neurones are mis-specified. A small population of V1 interneurones and SM neurones seem to be generated from narrow V1 and SM progenitor domains, respectively. Note that mis-specification profiles are different depending on to anteroposterior levels.

mutant. Therefore, we compared the expression patterns of the HD protein genes in the hindbrain at E11.5-12.5 between the wild type and *Pax6* mutant. We found that the expression patterns of Nkx2.2, *Nkx6.1*, *Irx3*, *Dbx1* and *Dbx2* (Fig. 2A-E) in the wild-type rat embryos were the same as reported in the mouse (Lu et al., 1992; Shoji et al., 1996; Bosse et al., 1997; Ericson et al., 1997), while all those in the *Pax6* mutant rat were markedly different (Fig. 2F-J). The Nkx2.2 domain, which faces the Pax6 domain, expanded dorsally (white arrowhead in Fig. 2F) as previously reported (Ericson et al., 1997; Osumi and Nakafuku, 1998). The dorsal boundary of *Nkx6.1* expression was slightly 'blurred' (double green arrowhead in Fig. 2G). *Irx3* expression markedly expanded ventrally into Nkx2.2 domain (red arrowhead in Fig. 2H). The ventral boundary of *Dbx2* expression was also 'blurred' (double green arrowhead in Fig. 2I). Cells expressing *Dbx1* were scattered ventrally (red arrowhead in Fig. 2J) and decreased in number when stained cells were counted in five sections from three embryos for both the wild type and mutant; 183.6±7.7 cells/section were positive for *Dbx1* in the wild-type hindbrain, while 101.8±7.9 cells/section were positive in the mutant (*t*-test; $P<0.001$).

Fig. 2K,L illustrates schematically the expression patterns of the HD protein genes in the hindbrain of the wild-type and *Pax6* homozygous embryos. Expression domains of *Irx3* and *Dbx1* (class I HD code genes) extended ventrally. The ventral expression boundary of *Dbx2*, another class I HD code gene, became blurred. Expression of Class II HD code genes also changed in the mutant; Nkx2.2 domain expanded dorsally, and the boundary between *Dbx2* and *Nkx6.1* was indistinct. As a result, formation of the progenitor domains was severely perturbed in the *Pax6* mutant. The progenitor domains for V1 interneurones and SM neurones, which are defined by expression boundaries of *Dbx1*/*Dbx2* and *Irx3*/Nkx2.2, respectively, became narrower. This explains very well why a very small number of V1 interneurones and SM neurones were produced in the *Pax6* mutant. By contrast, progenitor domains for BM neurones, V2 interneurones and V0 interneurones became expanded. This is consistent with the observation that the number of V2 interneurones increased in the mutant rat.

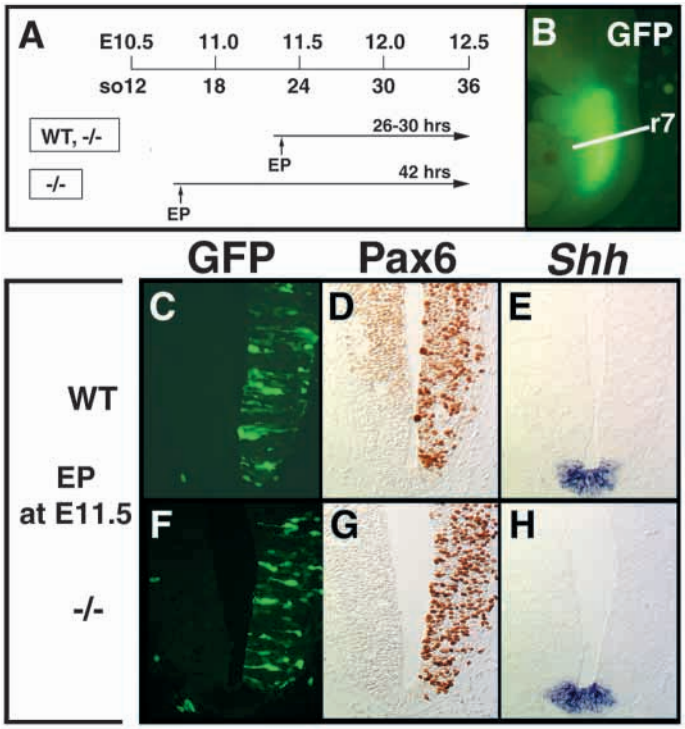

Fig. 3. Strategy of *Pax6* misexpression in cultured rat embryos by electroporation. (A) Experimental schedules of electroporation (EP) into cultured wild-type and *Pax6* homozygous mutant embryos starting from early E11.5 (22-somite stage) and E10.75 (16-somite stage). Electroporated embryos were cultured up to the stage corresponding to E12.5. (B) The electroporated area is monitored by GFP at harvest. White line indicates r7 level. (C-H) Exogenous *Pax6* was transfected into r7 of the wild-type embryo (C-E) and *Pax6* mutant (F-H) with *GFP* expression vector at E11.5, and these embryos were cultured up to E12.5 stage. After detection of *Shh* transcripts by in situ hybridisation (E,H), GFP protein is detected on the same sections by the antibody (C,F). *GFP* is transfected on the right side. (D,G) Adjacent sections of C and F, respectively. (D) Exogenous Pax6 protein is detected on the right, and endogenous Pax6 protein is detected on both sides. (G) In the *Pax6* mutant, only exogenous Pax6 protein is detected on the right side, consistent with GFP expression. (E,H) When *Pax6* is transfected in the region excluding the floor plate, endogenous expression of *Shh* is unaffected in the wild type and *Pax6* mutant.

Altered expression of HD code genes by misexpression of exogenous *Pax6*

As HD code gene expressions were altered in the loss-of-function condition of *Pax6*, we next examined the effects of *Pax6* gain-of-function on the expression of the HD code genes. *Pax6* expression vector (*pCAX-mPax6*) and *GFP* expression vector (*pCAX-GFP*) were co-transfected into the hindbrain of wild-type and *Pax6* mutant embryos by electroporation at 22-somite stage (early E11.5) in the rat embryos. These electroporated embryos were cultured for 26-30 hours up to the stage corresponding to 35- to 36-somite stage (E12.5; see Fig. 3A). We analysed embryos transfected on the right side of the hindbrain excluding the floor plate (Fig. 3C-H). In this electroporation, the expression of *Shh* was not affected (Fig. 3E,H).

In the wild-type hindbrain, *Pax6* overexpression (Fig. 4A) repressed *Nkx2.2* expression (bracket in Fig. 4B). By contrast, expression of *Irx3, Dbx2* and *Dbx1* were ectopically induced on the transfected side (arrowheads in Fig. 4D-F). These effects of *Pax6* overexpression were similarly observed in the *Pax6* mutant background (Fig. 4G-L). In the region where exogenous *Pax6* is expressed (Fig. 4G), *Nkx2.2* expression was downregulated (bracket in Fig. 4H), but *Nkx6.1* expression did not seem different (Fig. 4I). Ectopic induction of *Dbx2* and *Dbx1* expression was also observed (arrowheads in Fig. 4K,L). Induction of *Irx3* could not be detected in the *Pax6* mutant hindbrain, as *Irx3* expression had already shifted ventrally (Fig. 4J). Taken together, these results indicate that *Pax6* represses the expression of class II HD code genes such as *Nkx2.2*, while it induces the expression of class I HD code genes such as *Irx3, Dbx2* and *Dbx1*.

Rescue of *En1* and *Islet2* in the *Pax6* mutant by *Pax6* misexpression

We further investigated the effects of *Pax6* transfection on the expression of subtype specific markers of ventral neurones. As described above, *En1*-positive and *Islet2*-positive cells were mostly missing in the *Pax6* mutant (Fig. 1I,Q). We thus asked whether *Pax6* transfection could rescue this defect. First, we transfected *Pax6* expression vector into the hindbrain of wild-type embryos at 22-somite stage (early E11.5) when expression of *Islet2*, HB9/MNR2 and *En1* was undetectable (see Fig. 1A). The electroporated embryos were cultured for 26-30 hours up to the stage corresponding to E12.5. In the electroporated side at r7 level (Fig. 5A), ectopic expression of *Evx1* (Fig. 5E), but not of *Chx10* and *En1* (Fig. 5C,D), was observed. Ectopic expression of *Evx1* could be explained by the induction of *Dbx1* (see Fig. 4F), as *Dbx1* transfection induced Evx1 expression (Pierani et al., 2001). We also noticed fewer cells expressing *Islet2* (Fig. 5B) and HB9/MNR2 (data not shown) in response to *Pax6* overexpression. Thus, *Pax6* overexpression did alter the specification of neuronal subtypes.

Then we analysed the effects of exogenous *Pax6* in the mutant background. *Pax6* electroporation (Fig. 5F) at early E11.5 did not induce *Islet2* expression (Fig. 5G). However, we did observe expression of *En1* in the *Pax6* mutant hindbrain electroporated with exogenous *Pax6* (arrowheads in Fig. 5I), although *En1* expression was not observed at the r7 level in E12.5 mutant hindbrain (left side in Fig. 5I; also see Fig. 1B). The number of *En1*-positive cells was far less than the number in wild type, but similar to that in the *Pax6* mutant at E13.0 (see Fig. 1I). Importantly, these *En1*-positive cells were never seen in ectopic positions, suggesting that Pax6 is not sufficient for promoting development of V1 interneurones. *Chx10* expression was upregulated in the *Pax6* mutant (left side in Fig. 5H; also see Fig. 1H), but decreased in the electroporated area (right side in Fig. 5H). We sometimes observed *Chx10*-positive cells in ectopic positions (arrowhead in Fig. 5H). We also found a small number of cells expressing *Evx1* in normal and ectopic positions (arrow and arrowheads in Fig. 5J, respectively). As mentioned above, *Evx1*-positive cells never emerged at r7 level by E13.5 in our analysis (see Fig. 1B). Therefore, exogenous *Pax6* electroporated at early E11.5 induced *Evx1* expression, but the number of *Evx1*-positive cells was far less than that in normal development.

As differentiation to SM neurones started in the wild-type

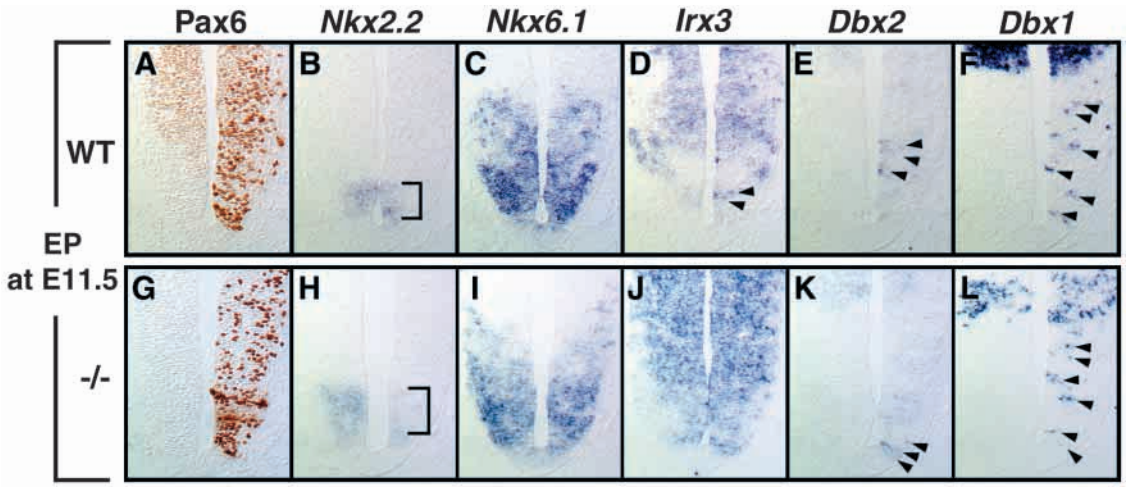

Fig. 4. Alteration of HD protein gene expressions by *Pax6* misexpression. (A-F) *pCAX-mPax6* was introduced into the hindbrain of wild-type embryos at 22-somite stage, and the embryo was cultured for 26 hours. Serial sections obtained from the same electroporated embryo. Exogenous Pax6 is detected in the right side of the hindbrain at a high level, while endogenous Pax6 is seen in both sides (A). Note repression of *Nkx2.2* expression (bracket in B) and ectopic expression of *Irx3*, *Dbx2* and *Dbx1* induced in the area where *pCAX-mPax6* is transferred (arrowheads in D-F). Expression of *Nkx6.1* is not changed by *Pax6* overexpression (C). (G-L) *pCAX-mPax6* was introduced into the hindbrain of *Pax6* mutant embryos at 22-somite stage, and the embryo was cultured for 30 hours (corresponding to E12.5) as neuronal differentiation is slightly delayed in the mutant. Serial sections obtained from the same electroporated embryo. Exogenous Pax6 is detected in the right side at a high level (G). Note that expression of *Nkx2.2* is downregulated (bracket in H), and ectopic expression of *Dbx2* and *Dbx1* is observed in the electroporated area (arrowheads in K,L). Expression of *Nkx6.1* seems unchanged by *Pax6* electroporation into the mutant hindbrain (I). Induction of *Irx3* is not detected, as *Irx3* expression shifts ventrally in the *Pax6* mutant hindbrain (J).

hindbrain at E11.5 (Fig. 1A), electroporation at early E11.5 might be too late to rescue *Islet2* and HB9/MNR2 expression. Therefore, we next electroporated exogenous *Pax6* into the hindbrain of E10.75 *Pax6* mutant embryo, and analysed the results after 42 hours culture (corresponding to E12.5). Although the efficiency of gene transfer at E10.75 was very low (Fig. 5K), in the area where *Pax6* was electroporated (Fig. 5K,L) a small number of *Islet2*-positive and HB9/MNR2-positive cells were detected at the ventral hindbrain (Fig. 5M-P). It was at E12.75-E13.0 that small populations of cells expressing *Islet2* and HB9/MNR2 were observed at r7 in the *Pax6* homozygous embryos (Fig. 1Q,R). Thus, the induction of *Islet2* and HB9/MNR2 expression was likely to be caused by exogenous *Pax6* expression. The induction of *Islet2* and HB9/MNR2 was only seen in a small number of cells (compare Fig. 5N,P with Fig. 1Q,R). Importantly, expression of these markers were never observed ectopically out of the positions where SM neurones normally exist, again suggesting that Pax6 function is not sufficient to induce these neurones.

In summary, these results of the overexpression experiments together with the loss-of-function data suggest that *Pax6* plays a crucial role in establishing V1 and SM progenitor domains and in subsequent differentiation of V1 and SM neurones.

Expression of Shh signalling molecules in the *Pax6* mutant hindbrain

What kind of mechanism is involved in progenitor domain formation? Expression of HD proteins in the spinal cord is influenced by graded action of Shh (Briscoe et al., 2000). Moreover, in the telencephalon of *Pax6* mutant mice, the domain expressing *Shh* expanded dorsally compared to that of the wild type (Stoykova et al., 2000). However, the expression patterns of *Shh* were not altered in the hindbrain and spinal cord of *Pax6* mutant mice and rats (Fig. 6A,G) (Ericson et al., 1997; Osumi and Nakafuku, 1998). To explore whether altered expression of HD code genes in the *Pax6* mutant hindbrain is due to changes in Shh signalling, we examined the expression of Shh receptor *Patched1* (*Ptc1*) and Gli family genes, both of which are direct targets of Shh signal (Goodrich et al., 1996; Hynes et al., 1997; Lee et al., 1997; Sasaki et al., 1999).

In the wild-type rat hindbrain at E12.5, *Ptc1* expression was seen in the region that included all the progenitor cells of the ventral neurones mostly in a ventral-to-dorsal gradient, with the highest expression in SM progenitor domain (bracket in Fig. 6C). This reflects the gradient distribution of Shh protein because *Ptc1* is induced in response to Shh activity (Goodrich et al., 1996). In the *Pax6* mutant, the overall expression pattern of *Ptc1* was not drastically changed, but the strong expression in the ventral region was not seen (Fig. 6I).

Expressions of *Gli1* and *Gli2* were detected at high level in the SM progenitor domain (bracket in Fig. 6D,E), and the ventral limits of the domains are adjacent to the dorsal limit of *Nkx2.2* domain (black arrowhead in Fig. 6B,D,E). The dorsal limit of *Gli1* and *Gli2* domains corresponded to that of *Dbx1* domain (red arrowhead in Fig. 6D-F). In the hindbrain of *Pax6* mutant, *Gli1* and *Gli2* were similarly expressed in the region between dorsal limits of *Nkx2.2* and *Dbx1* (green and red arrowheads, respectively, in Fig. 6H,J-L), but the high levels of *Gli1* and *Gli2* expression in the ventral region decreased. We also examined the expression patterns of a co-receptor of Shh, *Smoothened* (*Smo*) (Stone et al., 1996). In the hindbrain, *Smo* was expressed in the entire ventricular zone within the hindbrain, and the expression pattern was not altered in the *Pax6* mutant (data not shown). Thus, altered expressions of Shh signalling molecules were restricted to the progenitor domain of SM neurones in the *Pax6* mutant hindbrain, while expression

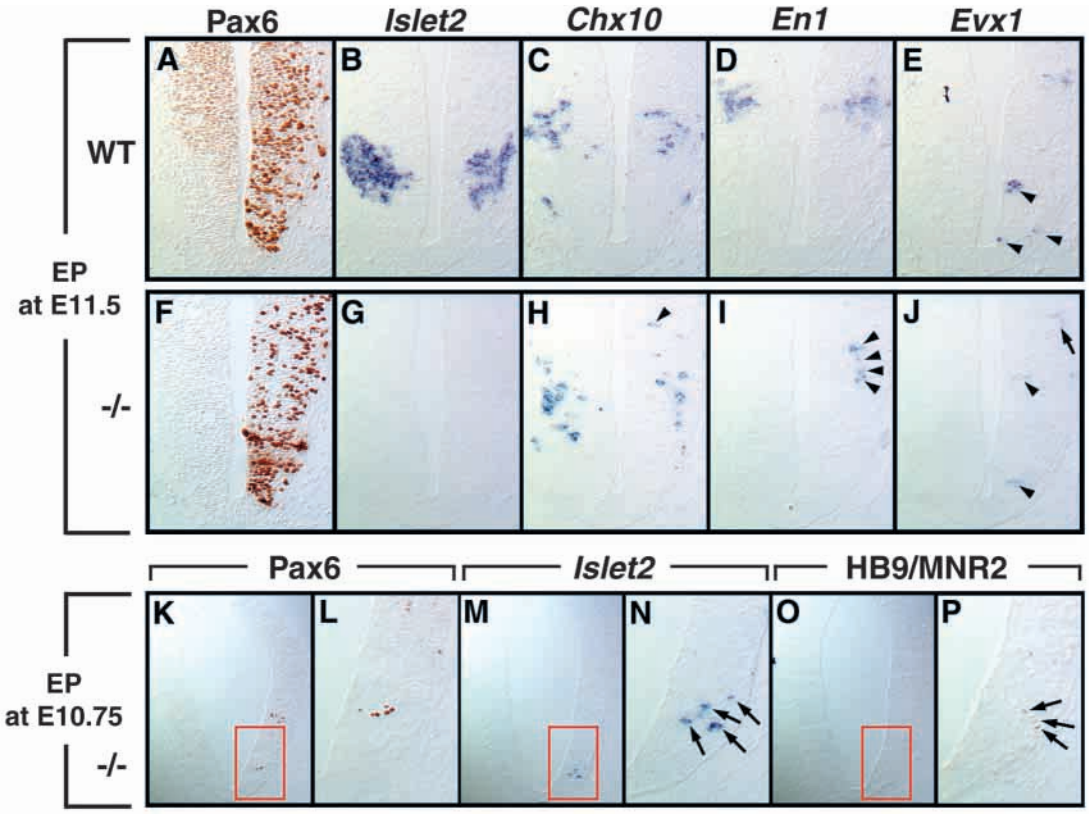

Fig. 5. Induction of *En1* and *Islet2* at r7 level in the *Pax6* mutant by exogenous *Pax6*. (A-E) *pCAX-mPax6* was introduced into the hindbrain of wild-type embryos at the 22-somite stage, and the embryos were cultured for 26 hours (corresponding to E12.5). (A) The same photograph as in Fig. 4A showing distribution of Pax6 protein. (B-E) These sections are adjacent to those shown as Fig. 4A-F, which were obtained from the same embryo. Ectopic expression of *Chx10* and *En1* is not observed (C,D), while *Islet2* expression is relatively downregulated (B). Ectopic expression of *Evx1* is observed (arrowheads in E) in the region where *Dbx1* is ectopically expressed (compare with Fig. 4F). (F-J) *pCAX-mPax6* was introduced into the hindbrain of *Pax6* mutant rat embryos at 22-somite stage, and the embryos were cultured for 30 hours (corresponding to E12.5), as neuronal differentiation is slightly delayed in the mutant. (F) The same photograph as in Fig. 4G showing Pax6 expression. (G-J) These sections are adjacent to those shown as Fig. 4G-L, which were obtained from the same embryo. Although *Islet2*-positive cells are undetectable in both unelectroporated and electroporated sides (G), expression of *En1* is recovered in the area where *pCAX-mPax6* was electroporated (arrowheads in I). (H) The number of *Chx10*-positive cells is relatively decreased but sometimes observed at an ectopic position (arrowhead). (J) *Evx1*-positive cells are observed not only in the normal position (arrow) but also in ectopic positions (arrowheads), where *Dbx1* is ectopically expressed (compare with Fig. 6L). (K-P) *pCAX-mPax6* was introduced into the hindbrain of *Pax6* mutant rat embryos at E10.75, and the embryos were cultured for 42 hours (corresponding to E12.5). (L,N,P) Higher magnifications of K,M,O, respectively. By electroporation into E10.75 *Pax6* mutant hindbrain, a small number of SM neurones expressing *Islet2* (M, N) and HB9/MNR2 (O,P) emerge in the area where *Pax6* is transfected (K,L).

patterns of these molecules were unchanged in the dorsal regions, including the progenitor domains for V2, V1 and V0 interneurones.

Cell death and cell proliferation in the *Pax6* mutant hindbrain

The impaired progenitor domain formation could be due to altered cell kinetics in the *Pax6* mutant. That is, the unstable narrow V1 and SM progenitor domains in *Pax6* mutant may be resulted from the change in cell death/proliferation in these and flanking domains. Thus, we investigated the relationship between cell death/proliferation and individual progenitor domains.

To detect apoptotic cells, we performed TUNEL staining at E12.5 stage (Fig. 7A,C) and counted the number of TUNEL-positive cells per section within the ventral region, including *Dbx1* domain. The mean number of TUNEL-positive cells in the wild-type hindbrain (9.4±1.1/section, ±s.e.m., n=10) was not significantly different from that in the *Pax6* mutant (12.3±1.3/section, n=10, t-test; P>0.1, Fig. 7E). Moreover, apoptotic cells were observed in random regardless of the progenitor domains.

Next, we performed BrdU pulse labelling, which detected cells in S-phase. The labelling index (percentage of $BrdU^+/DAPI^+$ cells per section) of the wild-type hindbrain was 27.0±0.32% (n=10, means±s.e.m.), and 31.1±0.57% (n=10) in the *Pax6* mutant (Fig. 7F). Although the ratio of S-phase cells was slightly higher in the *Pax6* mutant (t-test; P<0.001), there were no increase or decrease of BudU-positive cells in particular progenitor domains (Fig. 7B,D).

These results indicate that the disturbance of the progenitor domains in the *Pax6* mutant hindbrain is not attributed to change of cell death or proliferation in the specific progenitor domains.

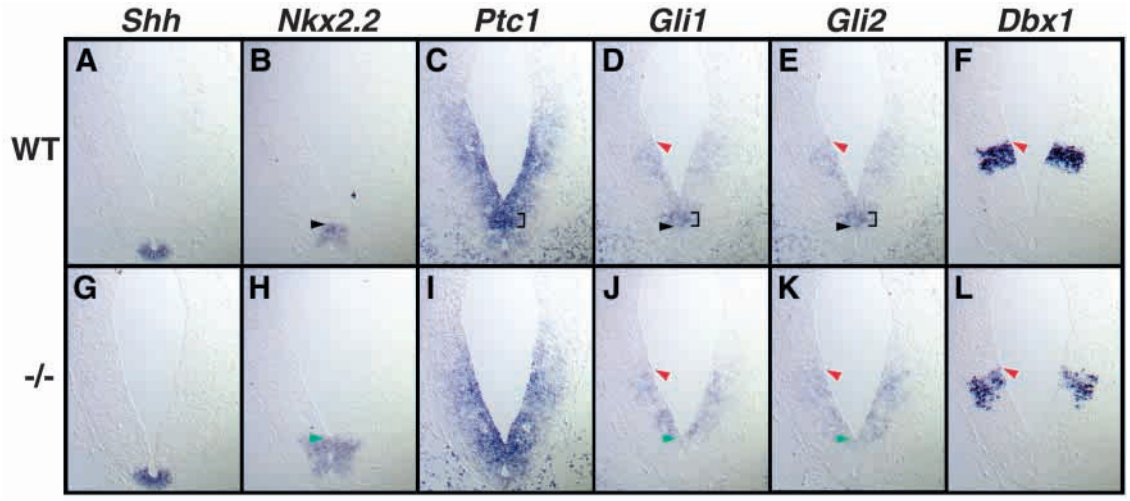

Fig. 6. Expression patterns of Shh signalling molecules in the *Pax6* mutant hindbrain. Adjacent sections of E12.5 wild-type (A-F) and *Pax6* mutant (G-L) hindbrains at r7 level. In the *Pax6* mutant hindbrain, *Shh* expression in the floor plate is no different from the wild-type (A,G). (B-F) In the wild type, *Ptc1* is expressed in all ventral progenitor cells with a ventral-to-dorsal gradient, and is especially strong in the SM progenitor domain (bracket in C). Ventral limits of *Gli1* and *Gli2* expression domains are adjacent to the dorsal limit of *Nkx2.2* domain (black arrowhead in B,D,E). The expression is missing in the BM progenitor cells expressing *Nkx2.2*, while it is detected at a high level in the SM progenitor cells (bracket in D,E). The dorsal limits of *Gli1* and *Gli2* expression domains correspond to that of *Dbx1* domain (red arrowhead in D-F). (H-L) In the *Pax6* mutant, strong *Ptc1* expression in the ventral region was not seen. *Gli1* and *Gli2* was expressed in the region between dorsal limits of *Nkx2.2* and *Dbx1* (green and red arrowheads, respectively, in Fig. 6H,J-L).

DISCUSSION

Pax6 regulates formation of correct progenitor domains

The *Pax6* mutant hindbrain has been characterised by the loss of SM neurones and V1 interneurones (Ericson et al., 1997; Osumi et al., 1997). However, in the present study, we found the emergence of a small number of SM neurones and V1 interneurones, although transiently, in the mutant hindbrain. Previous studies also reported that SM neurones indeed differentiate in the mutant spinal cord. Thus, *Pax6* does not appear to be required for specification of SM neurones. This implies that the function of *Pax6* may differ from other HD code genes.

The most important finding in this study is that loss of *Pax6* function leads to failure in formation of the correct progenitor domains within the ventricular zone. As illustrated in Fig. 2L, the expression boundaries of all HD protein genes are blurred and shifted in the *Pax6* mutant. Expression of *Nkx2.2* and *Dbx2* expands dorsally, while that of *Nkx6.1*, *Irx3* and *Dbx1* shifts ventrally. The altered expression patterns of the HD code genes in the *Pax6* mutant explain very well why a small number of V1 interneurones and SM neurones emerge; the progenitor domains for V1 interneurones and SM neurones, which are defined by the expression boundaries of *Dbx1*/*Dbx2* and *Irx3*/Nkx2.2, respectively, are formed as extremely narrow domains in the *Pax6* mutant. Emergence of these V1 and SM

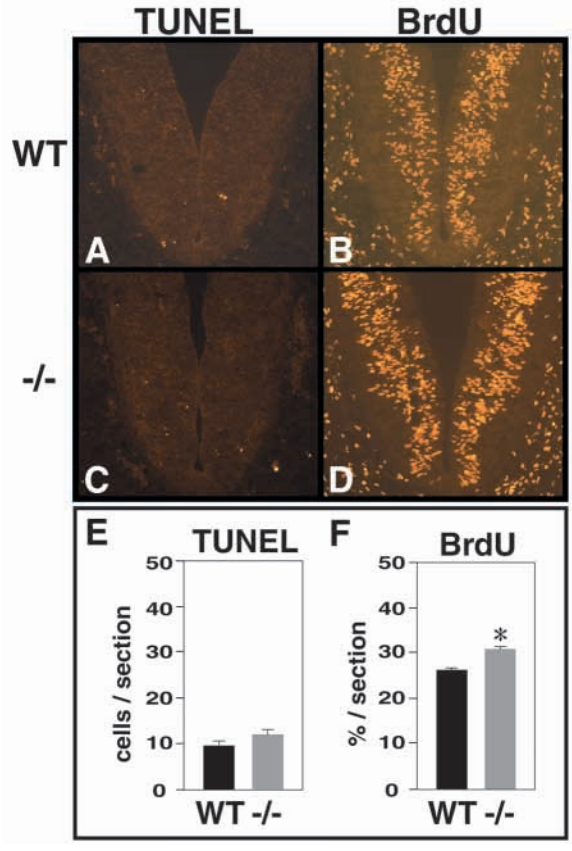

Fig. 7. Cell death and cell proliferation in the *Pax6* mutant hindbrain. Sections at r7 level of E12.5 wild-type (A) and *Pax6* mutant (C), and cultured wild-type (B) and *Pax6* mutant (D) embryos. (E,F) Comparison of TUNEL- (E) and BrdU- (F) positive cells per section within the ventral region, including the *Dbx1* domain using 10 sections from three embryos. (E) There is no significant difference in the number of apoptotic cells between the two groups. (B,D) Pulse labelling of BrdU was performed for embryos cultured for 20 minutes and BrdU labelled cells were detected with anti-BrdU antibody. The ratio of BrdU labelled cells is slightly increased in the *Pax6* mutant (*t*-test; **P*<0.001).

neurones may be transient (only for about ~10 hours) because these expression boundaries are not firmly maintained and such neurones will be diminished soon after. By contrast, progenitor domains for BM neurones, V2 interneurones and V0 interneurones became expanded. This is consistent with the observation that the number of V2 interneurones increased in the mutant rat, which differs from the results in the *Pax6* mutant mice reported by Ericson et al. (Ericson et al., 1997).

If *Pax6* is required for establishment of the progenitor domains in a correct manner, is it sufficient for progenitor domain formation? To answer this question, we performed overexpression of *Pax6* by electroporation into cultured rat embryos, and indeed rescued development of SM neurones and V1 interneurones in correct positions. The numbers of these rescued neurones were less than in normal development. The reason for this partial rescue may be that exogenous *Pax6* cannot fully re-establish the progenitor domains for SM neurones and V1 interneurones at the stage of electroporation. *Irx3*, the gene reported to repress SM fate in the chick spinal cord (Briscoe et al., 2000), was already expanded ventrally at the time of electroporation. Alternatively, exogenous *Pax6* could not cause a complete repression of the expanded expression of *Nkx2.2*. Taking these loss-of-function and gain-of-function studies together, we conclude that *Pax6* seems to regulate formation of the precursor domains in the hindbrain, thereby specify the fates of ventral neurones (Fig. 8).

Possible mechanisms for progenitor domain formation

The expression of *Nkx2.2* expands dorsally in the *Pax6* mutant, while overexpression of *Pax6* downregulated *Nkx2.2* expression (Ericson et al., 1997) (our present data). In the chick spinal cord, Pax6 and Nkx2.2 are reported to repress expression of each other (Briscoe et al., 2000; Muhr et al., 2001). Such mutual repression of Pax6 and other transcription factors is commonly observed in other regions of the developing brain (e.g. forebrain/midbrain boundary and cortex/LGE boundary), and is considered to be one of the mechanisms that defines brain territories (Matsunaga et al., 2000; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). However, *Pax6* also influences formation of the progenitor domains dorsal to SM progenitor domain within the hindbrain. Expression boundaries of *Nkx6.1*, *Irx3*, *Dbx2* and *Dbx1* are included within *Pax6* domain, and the boundaries of all these genes within Pax6 domain were very blurred in the *Pax6* mutant. What kind of other mechanism is therefore involved in setting the progenitor domains properly?

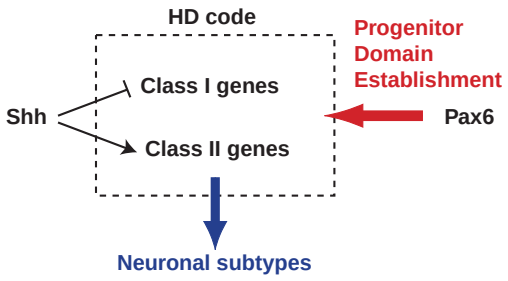

Fig. 8. The proposed model of Pax6 function in neuronal specification. The model indicates that Pax6 functions in establishment of progenitor domains in a correct manner, thereby regulating specification of neuronal subtypes. Shh, Sonic hedgehog.

The expression of HD code proteins is regulated by graded action of Shh released from the floor plate (Ericson et al., 1997; Briscoe et al., 2000). Therefore, we examined the expression patterns of the genes that are known to be direct targets of Shh signalling. We found that in the *Pax6* mutant, strong expression of *Ptc1*, *Gli1* and *Gli2* was eliminated in the ventral region corresponding to the SM precursor domain. Altered expression of Shh signalling molecules in the ventral region may suggest that establishment of SM progenitor domain may require *Pax6* function to respond properly to Shh signal. However, this might conversely be resulted from the perturbed progenitor domain formation.

The next possibility is different cell kinetics in individual progenitor domains. Previous studies have demonstrated that *Pax6* regulates cell proliferation in development of the diencephalon, telencephalon and retina (Caric et al., 1997; Warren and Price, 1997; Götz et al., 1998; Marquardt et al., 2001). The frequency and positions of dead cells were not different in the hindbrains of wild type and *Pax6* mutants. Although BrdU incorporation was slightly higher in the mutant, there was no relationship with specific progenitor domains. Therefore, it seems that overall disruption of progenitor domain boundaries in the mutant hindbrain is not explained by the alteration of cell death and proliferation.

Another possibility is that cell motility is accelerated in the neuroepithelium of the *Pax6* mutant. Notably, cell tracing analyses in chick embryos have revealed a widespread dispersal of neuroepithelial cells in the early stages, but such cell mixing becomes less obvious in later development when progenitor domains are established (Clarke et al., 1998; Erskine et al., 1998). There are accumulating data to suggest that Wnt signal regulates cell motility (Heisenberg et al., 2000; Jönsson and Anderson, 2001), and expression of *Wnt7b* is actually diminished in the *Pax6* mutant hindbrain (Osumi et al., 1997). It has also been reported that *Pax6* controls *R-cadherin* expression in the developing neocortex (Stoykova et al., 1997) (T. Inoue and N. O., unpublished). We have also found that the motility of neuroepithelial cells in the ventral telencephalon, where cadherin-6 is normally expressed, seems to be increased in *cadherin-6* deficient mice (Inoue et al., 2001). *Pax6* also controls granule cell migration in the cerebellum by modulating cytoskeletal components (Yamasaki et al., 2001). Collectively, *Pax6* may regulate, directly or indirectly, certain cell adhesion molecule(s) and/or cytoskeletal molecule(s) expressed in the neuroepithelium, thereby functioning in the establishment of the rigid precursor domains in the hindbrain.

Dose-dependent effect of Pax6 in specification of ventral neurones

The progenitor domain for SM neurones corresponds to the region where Pax6 expression is low (Ericson et al., 1997) (Fig. 2A). Overexpression of *Pax6* in the Pax6^{low} SM progenitor domain in the early E11.5 wild-type hindbrain repressed the production of *Islet2*-positive SM neurones. By contrast, exogenous *Pax6* in the E10.75 mutant embryos induced *Islet2*- and HB9/MNR2-positive cells. In the E10.75 hindbrain, Pax6 protein distribution is not seen in a gradient pattern (data not shown). Therefore, it is likely that differentiation of SM neurones is dependent on temporary different doses of Pax6.

In fact, it has been reported that *Pax6* influences eye formation and development of the diencephalic dorsal midline secretory radial glia in a dose-dependent manner (Schedl et al., 1996; Estivill-Torrús et al., 2001). Therefore, another interesting issue to investigate would be how much Pax6 is required for precise specification of SM neurones and perhaps other types of neurones.

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