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Identification of *Sox8* as a modifier gene in a mouse model of Hirschsprung disease reveals underlying molecular defect

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Abstract

Mice carrying heterozygous mutations in the *Sox10* gene display aganglionosis of the colon and represent a model for human Hirschsprung disease. Here, we show that the closely related *Sox8* functions as a modifier gene for *Sox10*-dependent enteric nervous system defects as it increases both penetrance and severity of the defect in *Sox10* heterozygous mice despite having no detectable influence on enteric nervous system development on its own. Sox8 exhibits an expression pattern very similar to Sox10 with occurrence in vagal and enteric neural crest cells and later confinement to enteric glia. Loss of *Sox8* alleles in *Sox10* heterozygous mice impaired colonization of the gut by enteric neural crest cells already at early times. Whereas proliferation, apoptosis, and neuronal differentiation were normal for enteric neural crest cells in the gut of mutant mice, apoptosis was dramatically increased in vagal neural crest cells outside the gut. The defects in enteric nervous system development of mice with *Sox10* and *Sox8* mutations are therefore likely caused by a reduction of the pool of undifferentiated vagal neural crest cells. Our study suggests that *Sox8* and *Sox10* are jointly required for the maintenance of these vagal neural crest stem cells.

Keywords: Sry; High-mobility-group; Sox10; Enteric nervous system; Hirschsprung disease; Megacolon

Introduction

The neural crest forms a migratory and multipotential cell population in the early vertebrate embryo which gives rise or contributes to many different organ systems (Le Douarin and Kalchheim, 1999). Accordingly, developmental defects of the neural crest are diverse. With a frequency of approximately 1 in 4500 newborns, Hirschsprung disease (HSCR or aganglionic megacolon, MIM 142623) is one of the more common of these neurocristopathies (Chakravarti, 2001). Hirschsprung disease is an intestinal disorder in which the enteric nervous system (ENS) fails to innervate the distal part of the colon over variable distances leading to intestinal obstruction and chronic constipation in affected individuals. Primarily affected are cells derived from the vagal neural crest which invade the foregut mesenchyme at the level of the esophagus, migrate as enteric neural crest cells in a caudal direction through the entire length of the gut and form the ENS (Newgreen and Young, 2002).

Mutations in several genes lead to Hirschsprung disease. Some of the identified mutations are sporadic, others are inherited. The genetics of Hirschsprung disease are complex with monogenic and oligogenic cases, syndromic and non-syndromic forms as well as dominant and recessive inheritance patterns (Chakravarti, 2001). The gene for the c-RET receptor tyrosine kinase is most frequently affected (Edery et al., 1994). Mutations in this gene alone or in combination with alterations in two other loci account for approximately half of all congenital Hirschsprung cases (Gabriel et al., 2002). In addition to its complex genetics, Hirschsprung disease also exhibits strong phenotypic variability pointing to considerable influences from environment and modifying genetic factors.

Additional genes in which mutations have been identified that lead or contribute to Hirschsprung disease are other

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components of the c-RET signaling cascade including the paracrine glial cell line-derived neurotrophic factor (*GDNF*) (Angrist et al., 1996; Salomon et al., 1996). Analogously, mutations in both the gene for endothelin-3 (*EDN3*) and the gene for endothelin receptor B (*EDNRB*) can cause Hirschsprung disease (Edery et al., 1996; Puffenberger et al., 1994) showing that the genetic defect can be autonomous to the cells of the ENS (as is the case for receptor mutations) or mediated by the gut environment and thus non-autonomous (as for ligand mutations).

Hirschsprung disease can also be caused by alterations in genes whose products directly regulate the pattern of gene expression as transcription factors or co-factors in cells of the ENS or their precursors. Examples of such genes are *ZFHX1B* encoding the Smad-interacting protein 1 (SIP1), and *SOX10* encoding a high-mobility-group-box containing transcription factor of the Sox family (Cacheux et al., 2001; Pingault et al., 1998; Wakamatsu et al., 2001). *SOX10* alterations in Hirschsprung patients are usually heterozygous mutations that either lead to truncated, non-functional versions of the protein or to a complete loss of expression from the affected allele (Kuhlbrodt et al., 1998b). This haploinsufficiency points to sensitivity of the ENS to SOX10 dosage.

Within the Sox family of transcription factors, SOX10 forms a separate group with two closely related proteins, SOX9 and SOX8 (Bowles et al., 2000; Wegner, 1999). Whereas mutations of *SOX9* lead to autosomal sex reversal and the severe skeletal malformation syndrome Campomelic dysplasia (Foster et al., 1994; Wagner et al., 1994), no disease has so far been associated with *SOX8*. Analysis in mice revealed that Sox8 is co-expressed with either Sox9 or Sox10 in many tissues, including the male gonad, muscle, the central nervous system, early neural crest cells, and chondrocytes (Chaboissier et al., 2004; Cheung and Briscoe, 2003; Schepers et al., 2003; Schmidt et al., 2003; Sock et al., 2001; Stolt et al., 2004), arguing for functional redundancy among these three Sox proteins. This functional redundancy might also explain the lack of a severe phenotype in Sox8-deficient mice (Sock et al., 2001).

Spontaneous and targeted mutations have been identified or generated for many mouse orthologs of human Hirschsprung disease genes (for review, see Gershon, 1999). Most of the corresponding mouse mutants exhibit defects in the development of the ENS, and impressively confirm the link between defects in these genes and Hirschsprung disease. Nevertheless, the exact phenotype often differs between humans and the mouse model, usually due to different gene dosage requirements in both species. In mice with heterozygous *c-Ret* mutations, for instance, there are no apparent changes in the ENS (Schuchardt et al., 1994), despite the fact that heterozygous *c-RET* mutations are the most common cause of Hirschsprung disease in humans (Gabriel et al., 2002).

Mice with genetic alterations of the *Sox10* gene faithfully reproduce the Hirschsprung phenotype as well as the incomplete penetrance and phenotypic variability (Herbarth et al., 1998; Paratore et al., 2002; Southard-Smith et al., 1998). The penetrance varies between different genetic backgrounds; C57Bl/6J mice, for instance, are affected at a higher rate than C3HeB/FeJ mice (Southard-Smith et al., 1999). In diseased mice, there is an enormous phenotypic variation with aganglionosis affecting different lengths of the colon. Both phenotypic variability and incomplete penetrance point to the existence of so far poorly characterized genetic modifiers (Southard-Smith et al., 1999).

Here, we have exploited the availability of mice with targeted mutation in the *Sox10* and *Sox8* genes to show that *Sox8* functions as such a modifier. We also obtained evidence that both genes influence survival of early neural crest cells. As a consequence, ENS defects in mice with *Sox8* and *Sox10* deletions are primarily due to strongly increased rates of apoptosis in vagal neural crest cells. Thus, developmental defects that ultimately lead to Hirschsprung disease may occur in the neural crest prior to gut colonization.

Materials and methods

Animal husbandry, genotyping, BrdU labeling, and tissue preparation

Mice with a $Sox8^{lacZ}$ allele (Sock et al., 2001) were kept as heterozygotes or homozygotes in the presence or absence of a $Sox10^{lacZ}$ allele on a mixed C57Bl/6J:C3HeB/FeJ background (Britsch et al., 2001). For the generation of embryos and pups during this study, $Sox8^{+/lacZ}$ mice were crossed with $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ double heterozygotes. Genotyping was performed by PCR as described (Britsch et al., 2001; Sock et al., 2001). For BrdU labeling, pregnant mice were injected with 100 μg BrdU (Sigma) per gram body weight 24 h before embryo preparation (Stolt et al., 2003). Embryos (from 9.5 to 16.5 dpc) were obtained from staged pregnancies. After fixation in paraformaldehyde, 20 μm sections of genotyped, age-matched mouse embryos were generated on a cryotome, and used for immunohistochemistry. Alternatively, guts dissected from embryos were used for whole-mount immunohistochemistry or whole-mount X-Gal staining.

Immunohistochemistry and X-Gal staining

For immunohistochemistry, the following primary antibodies were used in various combinations: anti-Sox10 guinea pig antiserum (1:2000 dilution), anti-NOS rabbit antiserum (1:500 dilution, Alexis Biochemicals), anti-PGP9.5 rabbit antiserum (1:400 dilution, Biotrend), anti-B-FABP rabbit antiserum, (1:10,000 dilution, gift of C. Birchmeier and T. Müller, MDC, Berlin), anti-S100β rabbit antiserum (1:500 dilution, Dako), anti-β-galactosidase rabbit antiserum (1:500 dilution, ICN) or anti-β-galactosidase goat antiserum (1:500 dilution, Biotrend). The guinea

pig anti-Sox10 antiserum was generated against a purified bacterially expressed protein consisting of amino acids 181–233 and 308–400 of rat Sox10 (Stolt et al., 2003) and did not cross-react with the related Sox8 or Sox9. Secondary antibodies conjugated to Cy2 and Cy3 immunofluorescent dyes (Dianova) were used for detection. TUNEL assays were performed using the ApopTag Red In Situ Apoptosis Detection Kit (Q Biogene). Incorporated BrdU was detected on tissue sections using an Alexa-488 coupled mouse monoclonal antibody directed against BrdU (Molecular Probes) at a 1:20 dilution. X-gal stainings followed standard procedures. Samples were analyzed and documented using either a Leica TCS SL confocal microscope or a Leica inverted microscope (DMIRB) equipped with a cooled MicroMax CCD camera (Princeton Instruments, Stanford).

Preparation of acutely dissociated gut cell cultures and gut homogenates

Cell culture preparations of dissociated gut regions were done as described (Paratore et al., 2002). Briefly, dissected gut regions from embryos at 12.5 and 14.5 dpc were digested in 1 mg/ml collagenase type I (Worthington Biochemical, NJ, USA) in PBS for 30 min at 37°C. For guts obtained from 16.5-dpc-old embryos, 0.01% trypsin (Invitrogen) was additionally added. After addition of fetal calf serum (FCS) to a final concentration of 1%, cells were collected by centrifugation, triturated, and plated at suitable densities in duplicate wells onto fibronectin coated cover slips in Dulbecco's modified Eagle's medium containing 10% FCS. After 3 h, most cells were adherent and were fixed in paraformaldehyde. No differential cell adherence was observed in gut preparations from different genotypes. Cells on cover slips were immunostained, counterstained by DAPI, photographed and scored for marker expression. At least four cover slips from two different embryos were analyzed for each genotype and developmental stage.

Homogenates were prepared from freshly dissected guts and from the post-otic region of 9.5 dpc-old embryos that harbors the vagal neural crest using a Polytron PT1200 homogenizer. Faeces were removed from postnatal guts through repeated rinsing prior to homogenization. After normalization of protein amounts in the homogenates, β-galactosidase activities were determined using a chemiluminescent detection kit (Roche Biochemicals).

Results

Sox8 functions as a modifier gene of Sox10 in the ENS

In the past, we have generated mice in which the complete open reading frame of the *Sox8* gene and the *Sox10* gene were each replaced by a lacZ marker (Britsch et al., 2001; Sock et al., 2001). Sox10-deficient mice died during embryogenesis or at birth, and completely lacked the ENS. $Sox10^{+/lacZ}$ mice, on the other hand, were born at normal Mendelian ratios. However, approximately 20% of these mice developed a megacolon and died during the first 3 postnatal weeks. Sox8-deficient mice were viable, fertile, and without any gross phenotypical abnormalities apart from a reduced body weight. When intercrossing $Sox8^{lacZ/lacZ}$ mice with $Sox10^{+/lacZ}$ mice (Fig. 1A), we noticed a high early postnatal mortality affecting 33% of the progeny. Of the remaining animals, 76% were heterozygous for *Sox8* and 24% were heterozygous for both *Sox8* and *Sox10*. As a different set of experiments revealed equal numbers of both genotypes at birth (data not shown), double heterozygous animals were lost at significant rates postnatally.

Whether underrepresentation of double heterozygous mice was solely due to the previously described occurrence of a megacolon phenotype in a fraction of $Sox10^{+/lacZ}$ mice (Britsch et al., 2001) was analyzed in intercrosses between $Sox8^{+/lacZ}$ mice with $Sox10^{+/lacZ}$ mice (Fig. 1B). Again, we observed an increased postnatal mortality of 19%. All animals that were genotyped post-mortem were heterozygous for the $Sox10^{lacZ}$ allele and of variable *Sox8* genotype. The obtained genotype distribution differed in a statistically significant manner from expected Mendelian ratios. Of the four genotypes which should all appear at equal ratios, *Sox10* heterozygous mice were present in 20% less than expected numbers at the time of genotyping. Sox10 heterozygous pups mainly died during the 3rd postnatal week concomitant with the dietary changes during weaning. Postmortem analysis confirmed the presence of a megacolon in all analyzed $Sox10^{+/lacZ}$ mice (compare Figs. 1C and D). The affected segment, which in the absence of innervation remained contracted and failed to transport the gut content, seldomly reached beyond the colon (Fig. 1D). As a consequence, faeces accumulated in front of the stenotic segment and grossly distended the adjacent, normally innervated gut segment, leading to the so-called megacolon.

In contrast, double heterozygous littermates were lost at a much higher, statistically significant rate. Seventy-seven percent did not survive the first 3 postnatal weeks (Fig. 1B). Of these, the majority was already lost during the first 2 postnatal weeks. Although phenotypes varied substantially between affected double heterozygous mice, the aganglionotic segment often extended beyond the caecum for various lengths into the small intestine (Fig. 1E). Thus, a heterozygous loss of *Sox8* by itself is phenotypically inapparent, but leads to a dramatic aggravation of the ENS defect associated with a heterozygous loss of *Sox10*, both with regards to increased frequency and increased severity.

Sox10 and Sox8 exhibit an overlapping expression in the ENS

To analyze the basis of this phenomenon, we first compared expression patterns of both Sox proteins in the

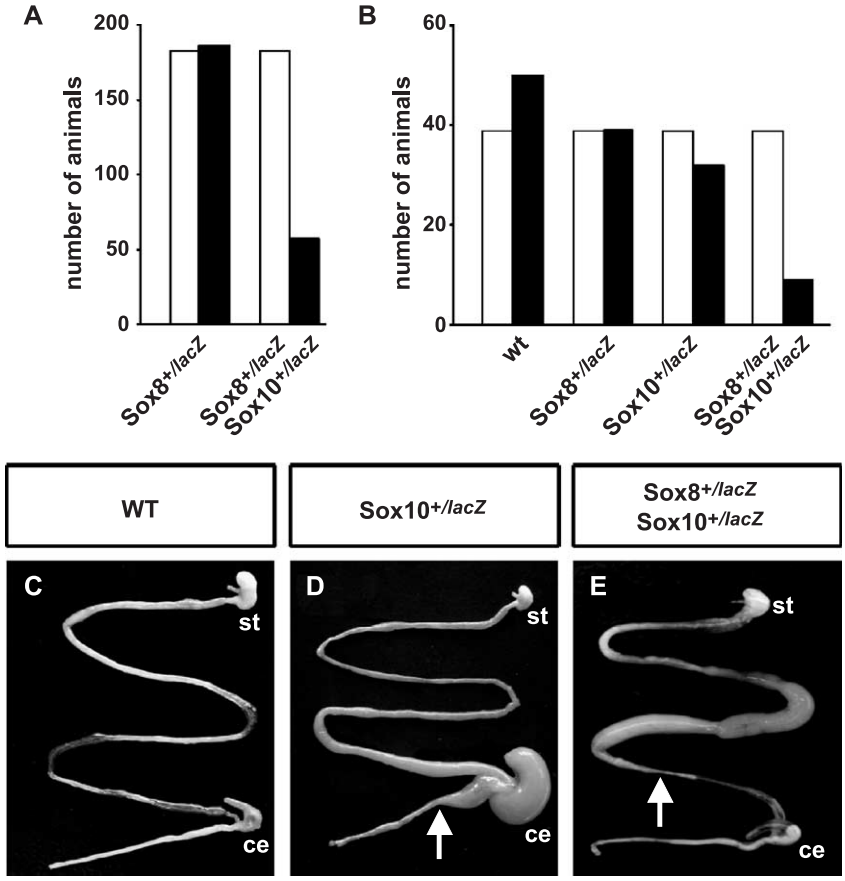

Fig. 1. Genotype distribution and phenotypic analyses of mice with combined *Sox8* and *Sox10* deletions. (A, B) Numbers of 3-week-old mice from various crossings sorted according to their genotype as indicated below the bars. In (A), $Sox10^{+/lacZ}$ mice were crossed with $Sox8^{lacZ/lacZ}$ mice. Of 362 newborns, 243 survived until weaning including 186 $Sox8^{+/lacZ}$ and 57 $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ mice. In (B), $Sox10^{+/lacZ}$ mice were crossed with $Sox8^{+/lacZ}$ mice. Of 160 newborns, 130 survived until weaning including 50 wildtype, 39 $Sox8^{+/lacZ}$, 32 $Sox10^{+/lacZ}$ and 9 $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ mice. Open bars, expected numbers; filled bars, obtained numbers. (C, D, E) Whole-mount preparations of gastrointestinal tracts from wildtype (C), $Sox10^{+/lacZ}$ (D), and $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ (E) mice. Guts in (C) and (D) were prepared from age-matched animals at postnatal day 18. Preparations for (E) was performed at postnatal day 12 because of earlier lethality. The arrow indicates the transition from stenotic to dilated gut segment. The stenotic region is aganglionotic and cannot perform peristaltic movements so that faeces accumulate in the gut region preceding the stenotic segment. The dilated segment, in contrast, has a morphologically normal enteric nervous system. st, stomach; ce, caecum.

developing ENS. Sox10 has previously been reported to occur in undifferentiated enteric neural crest cells where it is down-regulated upon neuronal specification (Paratore et al., 2002; Young et al., 2003). In contrast, Sox10 remains present in those enteric crest-derived cells acquiring a glial fate, and therefore becomes restricted to enteric glia as ENS development proceeds. We were able to reproduce these findings in two set-ups: (i) directly with antibodies against Sox10 on wildtype gut tissue (Figs. 2A–E) and (ii) indirectly with antibodies against β-galactosidase on $Sox10^{+/lacZ}$ tissue (Figs. 2F–J). The latter approach was made possible because the lacZ marker that had been inserted into the *Sox10* null allele faithfully recapitulated Sox10 expression (Britsch et al., 2001; Stolt et al., 2002). At 12.5 days post-coitum (dpc), both antibodies labeled PGP9.5-negative enteric neural crest cells (Figs. 2A, F). PGP9.5 is a ubiquitin hydrolase present in all neuronal precursors and differentiated neurons (Young and Newgreen, 2001). While Sox10/β-galactosidase- and PGP9.5-positive cells remained separate populations at later phases of ENS development (Figs. 2B, C, G, H), a steadily increasing fraction of Sox10/β-galactosidase-positive cells were co-stained with antibodies against the glial marker S100β (Figs. 2D, E, I, J).

Antibodies against β-galactosidase could also be used to follow Sox8 expression in the developing ENS. Again, this was made possible because of the lacZ marker insertion into the *Sox8* null allele (Sock et al., 2001). In essence, the Sox8-specific β-galactosidase expression in the gut of $Sox8^{+/lacZ}$ embryos closely resembled the Sox10-specific β-galactosidase pattern in the gut of $Sox10^{+/lacZ}$ embryos at all stages analyzed (compare Figs. 2K–O with Figs. 2F–J) indicating that Sox8 is also expressed in pluripotent enteric neural crest cells and enteric glia, but not in neuronal derivatives of the enteric neural crest.

To directly compare Sox10 and Sox8 expression in the developing ENS, we performed co-immunohistochemistry with antibodies against Sox10 and β-galactosidase on

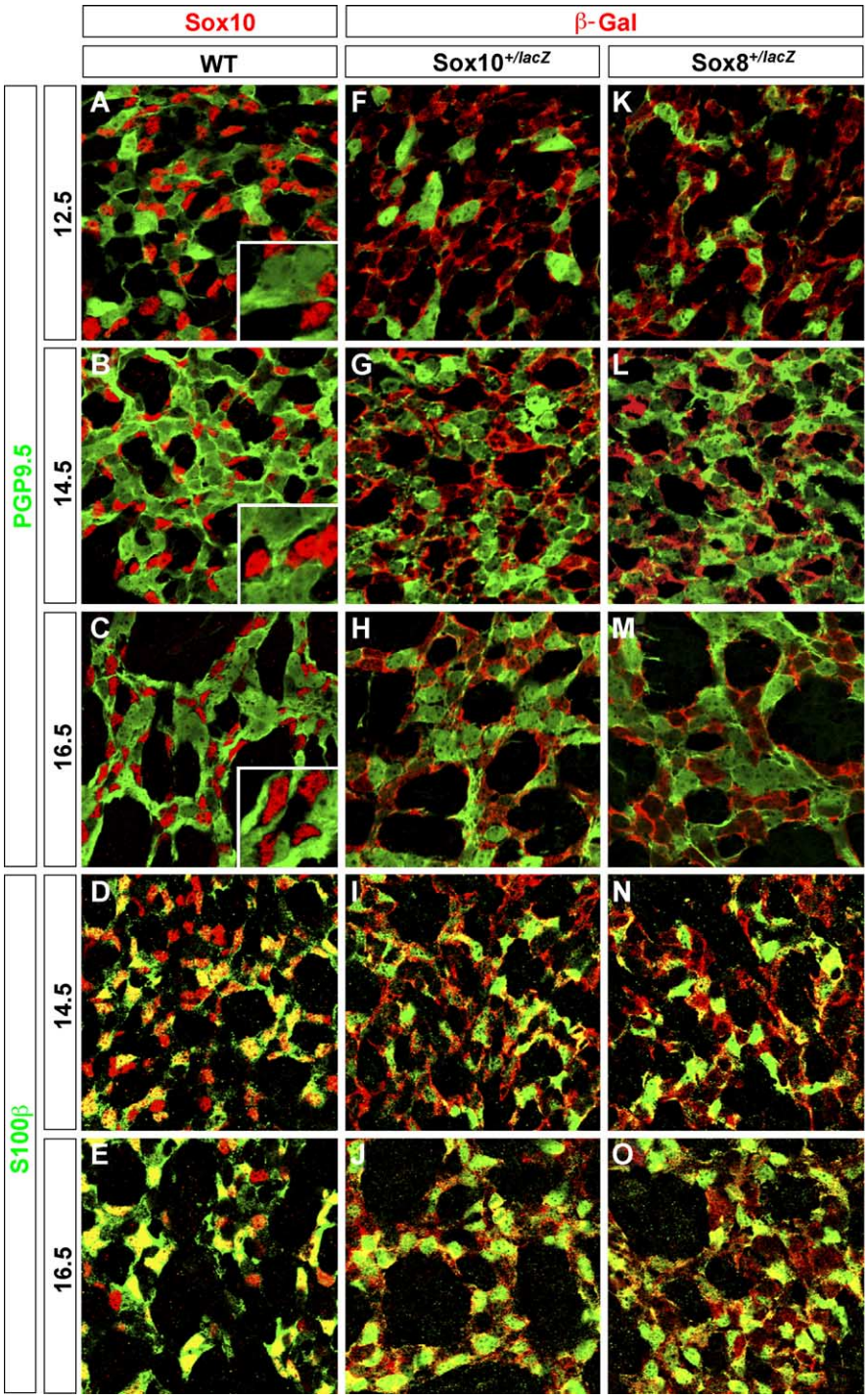

Fig. 2. Cell type-restricted expression of Sox10 and Sox8 in the embryonic ENS. Whole-mount immunohistochemistry was performed on guts from wildtype (A–E), Sox10$^{+/lacZ}$ (F–J) and Sox8$^{+/lacZ}$ (K–O) embryos at 12.5 dpc (A, F, K), 14.5 dpc (B, G, L, D, I, N) and 16.5 dpc (C, H, M, E, J, O) using antibodies against Sox10 or β-galactosidase (red) in combination with cell-type-specific antibodies including PGP9.5 (A–C, F–H, K–M), and S100β (D, E, I, J, N, O) (green). Magnifications (shown as insets in A–C) prove that Sox10-positive nuclei are separated from PGP9.5-positive cells by unstained cytoplasm. Pictures were taken from the midgut region using a Leica TCS SL confocal microscope.

Sox8$^{+/lacZ}$ embryonic gut. Nearly all cells with Sox10-labeled nuclei contained Sox8-specific β-galactosidase in their cytoplasm both at 12.5 dpc and at 14.5 dpc (Figs. 3A, B). For quantification, co-immunohistochemistry was also performed on acutely dissociated gut tissues of Sox8$^{+/lacZ}$ embryos (Fig. 3C). At 12.5 dpc, cells from stomach and midgut were separately quantified. At 14.5 dpc, quantification was performed on midgut and hindgut. In all cases, expression of both markers was intimately linked. Ninety-four percent to 98% of the Sox10-positive cells were

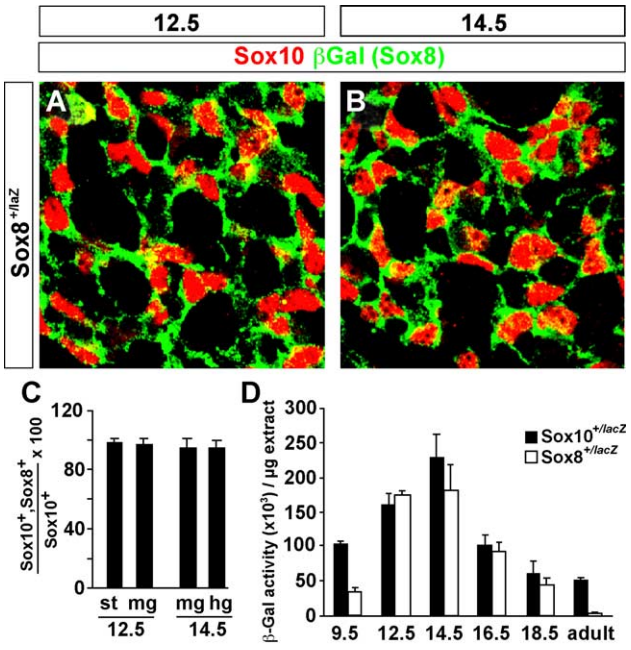

Fig. 3. Comparison of Sox10 and Sox8 expression in the embryonic ENS. (A, B) Whole-mount immunohistochemistry was performed on guts from $Sox8^{+/lacZ}$ embryos at 12.5 dpc (A) and 14.5 dpc (B) using antibodies against Sox10 (red) and β-galactosidase (green). Pictures were taken from the midgut region using a Leica TCS SL confocal microscope. (C) The fraction of Sox10-positive cells co-labeled by Sox8-specific β-galactosidase were determined in acutely dissociated gut regions from $Sox8^{+/lacZ}$ embryos at 12.5 and 14.5 dpc. On average, approximately 3000 cells in four separate cultures from two different embryos were counted. st, stomach; mg, midgut; hg, hindgut. (D) Quantification of β-galactosidase activities in $Sox10^{+/lacZ}$ (filled bars) and $Sox8^{+/lacZ}$ (open bars) vagal regions (9.5 dpc) and intestines (12.5, 14.5, 16.5, and 18.5 dpc) as well as in the adult colon. Activities are presented as relative light units × 10^3 per microgram gut extract.

positive for the Sox8-specific β-galactosidase (Fig. 3C). A similarly good correlation was also obtained for the reverse setup (data not shown). These analyses confirm that Sox8 and Sox10 are co-expressed during development of the ENS both in enteric neural crest cells and in forming enteric glia.

To compare expression levels for both Sox proteins, we quantified β-galactosidase activity in gut homogenates of $Sox8^{+/lacZ}$ and $Sox10^{+/lacZ}$ mice at various times of development. As the same lacZ reporter was inserted in similar manner into both *Sox8* and *Sox10* loci (Britsch et al., 2001; Sock et al., 2001; Stolt et al., 2002, 2004), β-galactosidase activity should yield an approximate measure for the relative expression levels of both genes. From 12.5 through 18.5 dpc, β-galactosidase activities were comparable for *Sox8* and *Sox10* and similarly decreased during late embryogenesis (Fig. 3D). In contrast, β-galactosidase activities differed significantly for both Sox genes in the early vagal crest and in the adult gut. At 9.5 dpc, β-galactosidase was expressed at 3-fold higher levels from the *Sox10* locus than from the *Sox8* locus in the post-otic region of the embryo that contained the vagal crest. In the adult, β-galactosidase activity was reduced to levels barely above background both in the colon and small intestine of $Sox8^{+/lacZ}$ mice (Fig. 3D and data not shown) indicating that *Sox8* might be no longer expressed in the adult gut. This agrees with our previous failure to detect *Sox8* expression in the adult gut by RT PCR analysis (Sock et al., 2001). In contrast, β-galactosidase activity was easily detected in $Sox10^{+/lacZ}$ mice because of the continued expression of Sox10 in enteric glia (Young et al., 2003).

ENS development is altered in mice with combined Sox8 and Sox10 mutations

As both Sox8- and Sox10-specific β-galactosidase was expressed in enteric neural crest cells, we used this marker for analysis of gut colonization and ENS development in mice with mutant *Sox8* and *Sox10* alleles. As evident from immunohistochemistry with Sox10 and PGP9.5 antibodies, ENS development in $Sox8^{+/lacZ}$ and in $Sox8^{lacZ/lacZ}$ mice was identical to the wildtype at all times (data not shown), consistent with the fact that we never observed a megacolon in mice of these genotypes. In wildtype mice and littermates with *Sox8* deletions, enteric neural crest cells had colonized most regions of the gut at 12.5 dpc with the migratory wavefront just past the caecum (Figs. 4A, B). Similar results were generally obtained for guts of $Sox10^{+/lacZ}$ embryos (Figs. 4D, E), indicating normal ENS development in most *Sox10* heterozygous embryos. However, a minor fraction of $Sox10^{+/lacZ}$ embryos displayed a retarded colonization with enteric neural crest cells being stalled before the caecum (data not shown). Thus, variability of the gut phenotype in *Sox10* heterozygous animals is already evident at this early stage of development.

When *Sox10* heterozygous animals were analyzed that had additionally lost one or two *Sox8* alleles, colonization of the gut was strongly delayed in most cases. In general, the wavefront of migrating enteric neural crest cells appeared least advanced in $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos, with most midgut regions still free of enteric neural crest cells (Figs. 4J, K). In many $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos, migratory delays were intermediary between those of $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos and the affected $Sox10^{+/lacZ}$ embryos (Figs. 4G, H). However, there was a significant overlap between phenotypes of $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos and $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos. Gut colonization in a severely affected $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryo could on occasion be indistinguishable from that in a weakly affected $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryo. It should also be noted that ENS development appeared normal in a few $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos in agreement with the fact that 23% of these animals do not develop a megacolon postnatally (Figs. 1A, B).

When neuronal markers such as PGP9.5 and NOS were used for the analysis instead of the enteric neural crest marker β-galactosidase, similar results were obtained, as neuronal commitment and differentiation already occur

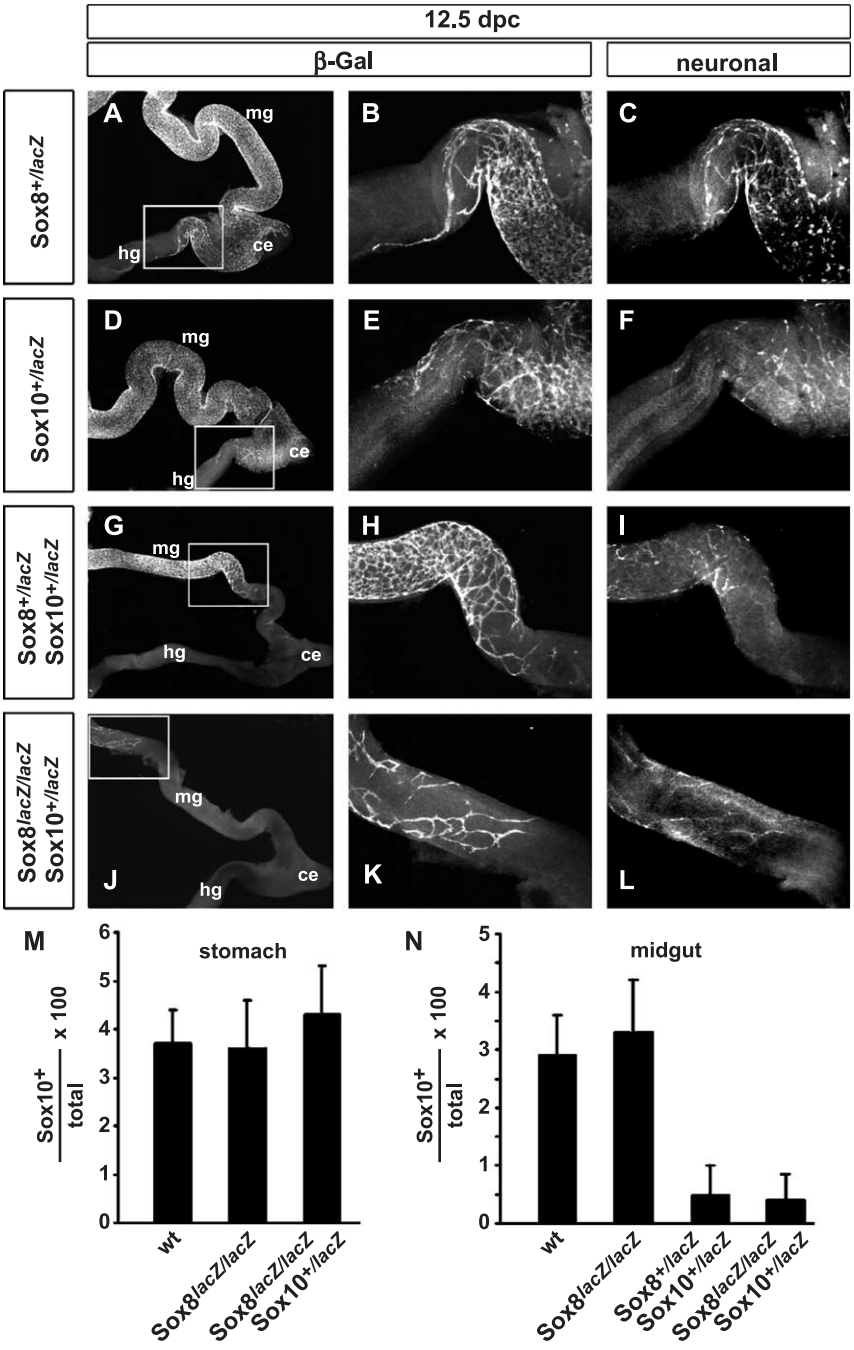

Fig. 4. ENS development in mice with mutant *Sox8* and *Sox10* alleles at 12.5 dpc. (A–L) Whole-mount immunohistochemistry was performed on guts from $Sox8^{+/lacZ}$ (A–C), $Sox10^{+/lacZ}$ (D–F), $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ (G–I), and $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ (J–L) embryos using antibodies against β-galactosidase (A, B, D, E, G, H, J, K) or the neuronal markers PGP9.5 and NOS (C, F, I, L). (B, E, H, K), magnifications of the respective boxed areas in (A, D, G, J). (M, N) Fraction of Sox10-positive cells in cultures of acutely dissociated stomach (M) and midgut (N). On average, approximately 3500 cells in four separate cultures from two different embryos were counted for each genotype. Data are presented as mean ± SEM. Differences to the wildtype were statistically significant for $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ and $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos in (N) as determined by the Student's *t* test ($P \leq 0.001$). Note that gut colonization was retarded more strongly in the $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos shown in (N) than in the one shown in (G).

closely behind the wavefront of migrating enteric neural crest cells (Conner et al., 2003). Interestingly, cells of neuronal identity could be detected in comparable distances from the wavefront of migrating enteric neural crest cells in all genotypes thus arguing that there is no genotype-specific delay in neurogenesis (Figs. 4C, F, I, L).

The total number of enteric neural crest cells was also quantified in cultures from acutely dissociated gut regions at 12.5 dpc using antibodies directed against Sox10 (Figs. 4M, N). In the stomach, these analyses failed to detect significant differences in the number of enteric neural crest cells between genotypes (Fig. 4M, and data not shown).

However, when enteric crest cells were quantified in the midgut, significant reductions to 13–17% of wildtype numbers became obvious for the analyzed $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ and $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos (Fig. 4N).

At 14.5 dpc, enteric neural crest cells had reached the terminal end of the hindgut in both wildtype embryos and embryos with Sox8 deletions (Figs. 5A–C). This was also the case for most $Sox10^{+/lacZ}$ embryos (Figs. 5D–F). However, in some Sox10 heterozygous embryos, colonization of the gut by enteric neural crest cells was clearly disturbed with the distal segment of the hindgut still devoid of enteric neural crest cells (Figs. 5G–I). The vast majority of embryos with additional deletions of one or two *Sox8* alleles showed a much stronger retardation. In some embryos of these genotypes, the wavefront of migrating enteric neural crest cells was found shortly before or behind the caecum at 14.5 dpc (Figs. 5J–L). In others, the midgut region had still received hardly any enteric neural crest cells (Figs. 5M–O). As already observed at 12.5 dpc, the gut phenotypes of $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ and $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos varied widely so that there was a broad phenotypic overlap between these two groups of embryos. However, the strongest affected animals were found among the $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos. Similar results were obtained in whole-mount immunohistochemistry, independent of whether enteric crest markers or neuronal markers were used (compare Figs. 5B, E, H, K, N with C, F, I, L, O).

A detailed quantification was performed on cultures of acutely dissociated midgut (including caecum) and hindgut (Figs. 5P, Q). In midgut and caecum, the relative number of Sox10-positive enteric neural crest cells and of NOS-positive inhibitory neurons was roughly comparable between all genotypes with the exception of $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos (Fig. 5P and data not shown). The strongly reduced number of enteric crest cells and neurons in the latter genotype corresponded to the defective midgut colonization frequently observed for $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos in whole-mount immunohistochemical analyses (Figs. 5M–O). As a consequence, we also failed to detect enteric neural crest cells or inhibitory neurons in the hindgut region of these embryos (Fig. 5Q). Compared to control littermates, Sox10-positive enteric neural cells and NOS-positive inhibitory neurons were also reduced in the hindgut region of $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos (Fig. 5Q). However, reductions of enteric neural crest cells and inhibitory neurons were proportionate, thereby confirming that there is no significant delay in neuronal differentiation in any of the genotypes analyzed.

Even at 16.5 dpc, many guts from $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos and all analyzed guts of $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos still exhibited clear defects in colonization by enteric crest cells as judged by X-gal staining (Figs. 6A–D). From comparing embryonic phenotypes at 14.5 and 16.5 dpc, it appeared that the wavefront of migrating enteric neural crest cells had not significantly advanced. Migration of these cells had at least not proceeded at any faster rate than longitudinal growth of the gut. Quantification of cells in acutely dissociated gut cultures showed absence of ENS cells for $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos in the distal ileum and beyond as well as strong reduction of ENS cells for most $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos in the distal colon (Fig. 6E). At this age, we also analyzed the percentage of ENS cells that were already specified to a glial fate by determining the fraction of Sox10-positive cells co-labeled by the early glial marker B-FABP (Fig. 6F). Interestingly, we found a significant reduction of double-positive cells in $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos indicating that determination of enteric neural crest cells to a glial fate is decreased in mice with combined mutations in Sox8 and Sox10. This decrease was not only observed in the distal colon in which overall ENS cell numbers were reduced, but also in the distal ileum in which ENS cell numbers appeared relatively normal in $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ mice. Therefore, reduced gliogenesis in mutant mice is not just a consequence of a reduced cell density in the ENS.

Increased apoptosis of vagal neural crest cells is a major determinant of the ENS defects in mice with combined Sox8 and Sox10 mutations

To determine the cause of the ENS defect in mice with combined Sox8 and Sox10 mutations, we also analyzed proliferation rates of enteric neural crest cells across the various genotypes (Figs. 7A, B). For this purpose, we prepared dissociated gut cultures from BrdU-labeled embryos and identified proliferating enteric neural crest cells as those cells, which were co-labeled with both anti-Sox10 and anti-BrdU antibodies. In general, proliferation rates of enteric neural crest cells were higher in more distal regions where active colonization of the gut was still ongoing. For instance, 24% of all enteric neural crest were BrdU-labeled at 12.5 dpc in the stomach of wildtype embryos and littermates with Sox8 deletions, whereas 59% were BrdU-labeled in the midgut region (Fig. 7A). The fraction of BrdU-labeled enteric neural crest cells had dropped to 33% in the distal midgut of wildtype embryos and littermates with Sox8 deletions at 16.5 dpc (Fig. 7B). Similarly decreasing rates were also observed for the hindgut. When proliferation was analyzed in $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos and $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos, very similar rates were detected with one exception. In $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos, there even was an increase in the percentage of BrdU-labeled enteric neural crest cells at 16.5 dpc in the colon, which in contrast to control embryos was not yet fully colonized (Fig. 7B). Clearly, proliferation rates of enteric neural crest cells in mice with combined Sox8 and Sox10 deletions were not lower than those in the wildtype. Compared to the wildtype, we also failed to detect significant increases in the number of TUNEL-positive cells in $Sox8^{lacZ/lacZ}$,

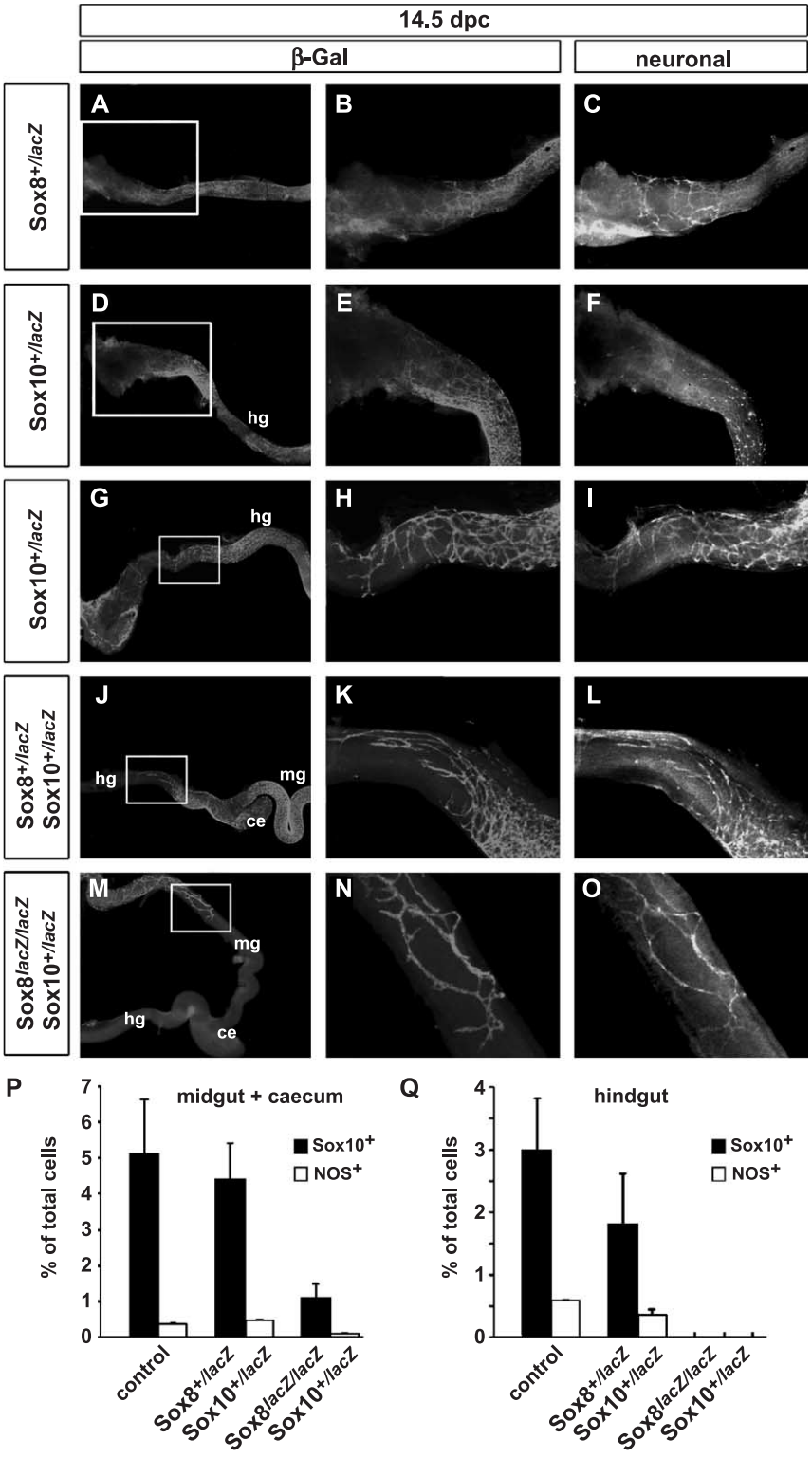

Fig. 5. ENS development in mice with mutant *Sox8* and *Sox10* alleles at 14.5 dpc. (A–L) Whole-mount immunohistochemistry was performed on guts from $Sox8^{+/lacZ}$ (A–C), $Sox10^{+/lacZ}$ (D–I), $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ (J–L), and $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ (M–O) embryos using antibodies against β-galactosidase (A, B, D, E, G, H, J, K, M, N) or neuronal markers PGP9.5 and NOS (C, F, I, L, O). (B, E, H, K, N), magnifications of the respective boxed areas in (A, D, G, J, M). (P, Q) Fraction of Sox10-positive (filled bars) and NOS-positive (open bars) cells in cultures of acutely dissociated midgut including caecum (P) and hindgut (Q). On average, approximately 2000 cells were counted for Sox10 and approximately 7000 cells for NOS in four separate cultures from two different embryos for each genotype. Data are presented as mean ± SEM. Differences to the wildtype were statistically significant for $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos in (Q) and $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos in (P, Q) as determined by the Student's *t* test ($P \leq 0.01$).

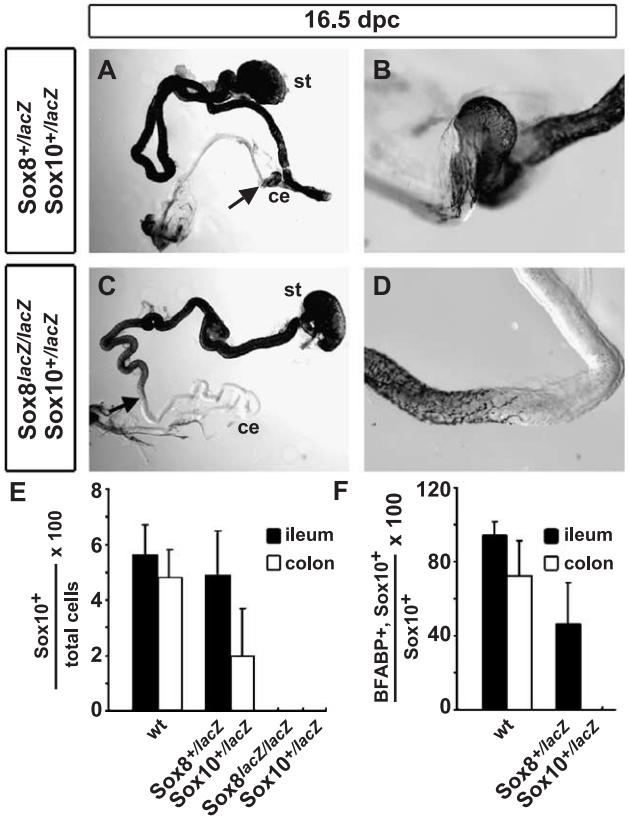

Fig. 6. ENS development in mice with mutant Sox8 and Sox10 alleles at 16.5 dpc. (A–L) Whole-mount X-Gal staining was performed at 16.5 dpc on guts from Sox8$^{+/lacZ}$, Sox10$^{+/lacZ}$ (A, B) and Sox8$^{lacZ/lacZ}$, Sox10$^{+/lacZ}$ (C, D) embryos. (B, D) Magnifications of the distalmost colonized areas (marked by arrows in A, C). (E) Percentage of Sox10-positive cells in cultures of acutely dissociated distal ileum (filled bars) and distal colon (open bars). Differences to the wildtype were statistically significant for Sox8$^{+/lacZ}$, Sox10$^{+/lacZ}$ embryos in the colon and for Sox8$^{lacZ/lacZ}$, Sox10$^{+/lacZ}$ embryos in both regions as determined by the Student's *t* test ($P \leq 0.001$). (F) Fraction of Sox10-positive cells co-labeled by B-FABP in acutely dissociated gut regions. Differences to the wildtype were statistically significant for Sox10$^{+/lacZ}$, Sox8$^{+/lacZ}$ embryos as determined by the Student's *t* test ($P \leq 0.001$). On average, approximately 4000 cells in four separate cultures from two different embryos were counted for each genotype.

Sox10$^{+/lacZ}$ and Sox8$^{+/lacZ}$, Sox10$^{+/lacZ}$ embryos from 12.5 dpc onwards in whole-mounts or dissociated gut cultures (data not shown). Rates of apoptosis were similar for enteric neural crest cells in the different genotypes.

Therefore, we extended our analysis to earlier times in embryogenesis. Specifically, we determined the behavior of vagal neural crest cells around the time of immigration into the foregut (for analyzed region of the embryo, see Fig. 7C). In wildtype, Sox8$^{+/lacZ}$ and Sox8$^{lacZ/lacZ}$ embryos, these vagal neural crest cells were readily seen entering the foregut at 9.5 dpc (Fig. 7D, and data not shown). Very few TUNEL-positive cells were detected in the stream of these migrating neural crest cells. In Sox10$^{+/lacZ}$ embryos, the number of TUNEL-positive neural crest cells was already higher (Fig. 7E). Interestingly, TUNEL-positive cells were primarily localized outside the gut arguing that vagal neural crest cells might be more sensitive to cell death before entering the gut than the enteric neural crest cells after entering.

TUNEL-positive vagal neural crest cells were even more increased in Sox8$^{lacZ/lacZ}$, Sox10$^{+/lacZ}$ embryos (Fig. 7F), and reached their maximum in Sox10-deficient embryos (Fig. 7G). In the latter, apoptosis was so high that neural crest cells failed to reach and enter the gut. Thus, the most obvious defect in embryos with combined Sox8 and Sox10 deletions is a dramatically increased death rate in the vagal neural crest that leads to a drastic depletion of the enteric neural crest precursor pool before immigration into the gut.

Discussion

Sox10$^{+/lacZ}$ mice model many features of Hirschsprung disease

Heterozygous *Sox10* mutations lead to aganglionosis of the colon with incomplete penetrance and significant phenotypic variability in humans and mice (Herbarth et al., 1998; Pingault et al., 1998; Southard-Smith et al., 1998). On the mixed C57Bl/6J:C3HeB/FeJ background used in this study, approximately 20% of the *Sox10$^{+/lacZ}$* mice developed a megacolon and did not survive weaning. Their colon proved aganglionic over variable distances. These similarities to the human condition indicate that Sox10 dosage effects are comparable in both species. Incomplete penetrance and variable phenotype furthermore point to the existence and influence of modifier genes as determinants of the disease. For that matter, mice with heterozygous *Sox10* mutations also offer a unique tool to identify such modifiers and explain their mode of action on a molecular basis. In this report, we have identified *Sox8* as such a modifier gene for *Sox10*. Whether Sox8 is identical to the modifier gene responsible for the different incidence of a megacolon in heterozygous Sox10 mice on a C57Bl/6J versus C3HeB/FeJ background (Southard-Smith et al., 1999) is not known.

Sox8 is a modifier gene for Sox10-dependent intestinal aganglionosis

Mice deficient for Sox8 have a reduced weight, but are viable and fertile (Sock et al., 2001). They do not show any sign of constipation or megacolon, and there is no increased early postnatal lethality. By histological and immunohistochemical criteria, the ENS is normal throughout the colon. Sox8-related ENS defects appear only on top of heterozygous Sox10 mutations. The mortality rate of 77% in Sox8$^{+/lacZ}$, Sox10$^{+/lacZ}$ mice is significantly above the approximately 20% observed for Sox10$^{+/lacZ}$ mice on the same mixed genetic background. Analysis of the gut of Sox8$^{+/lacZ}$, Sox10$^{+/lacZ}$ mice furthermore revealed that the aganglionic segment of

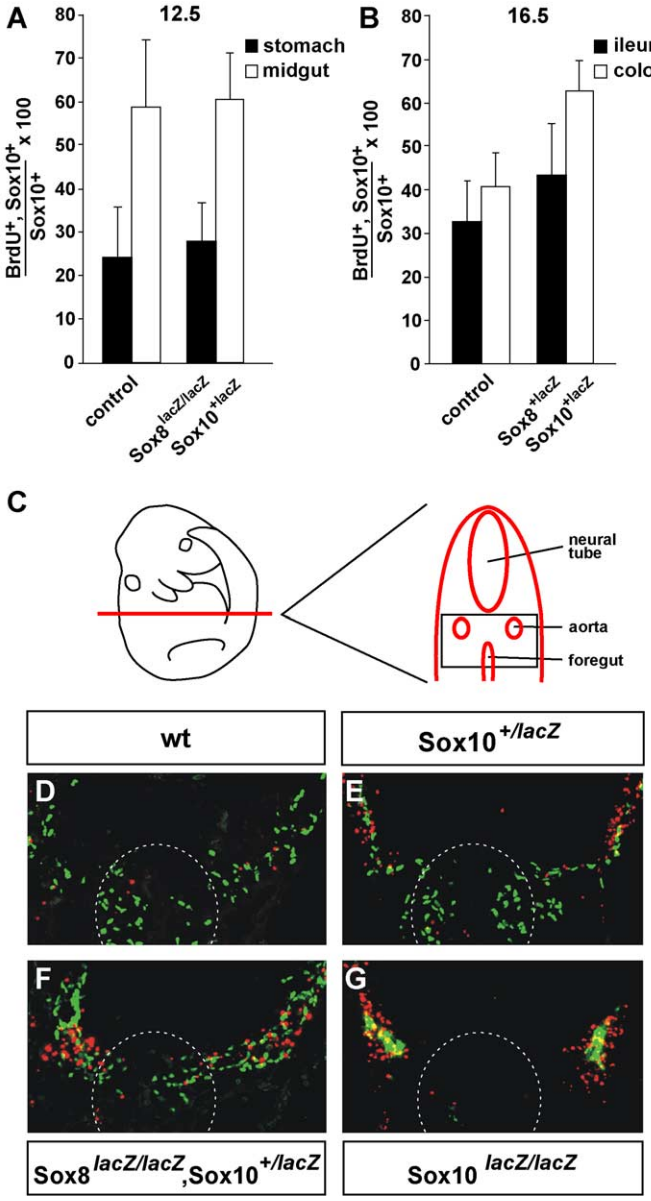

Fig. 7. Proliferation in the developing ENS and apoptosis in the vagal neural crest of mice with mutant Sox8 and Sox10 alleles. (A, B) Fraction of Sox10-positive cells co-labeled by BrdU in acutely dissociated cultures of stomach (filled bars in A), midgut (open bars in A), ileum (filled bars in B), and colon (open bars in B) of $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos, $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos and control littermates at 12.5 dpc (A) and 16.5 dpc (B). On average, approximately 2500 cells in four separate cultures from two different embryos were counted for each genotype. Statistically significant alterations in proliferation rates relative to the wildtype were detected only in the colon of $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ embryos as determined by the Student's t test ($P \leq 0.01$). (C–G) Immunohistochemistry was performed on vagal neural crest in post-otic transverse sections of 9.5-dpc-old embryos. Plane and position of section are indicated in (C). The area shown in (D–G) is marked by a black box. Wildtype (D), $Sox10^{+/lacZ}$ (E), $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ (F), and $Sox10^{lacZ/lacZ}$ embryos (G) were analyzed using antibodies against Sox10 (D–F) and β-galactosidase (G) (green) in combination with a TUNEL stain (red). The dotted line in (D–G) demarcates the region of the foregut mesenchyme.

the gut was on average longer than the one in affected $Sox10^{+/lacZ}$ mice and often extended beyond the colon far into the ileum. Thus, loss of *Sox8* not only increases penetrance, but also severity of the disease in $Sox10^{+/lacZ}$ mice. Arguing for a dosage effect, both parameters of the ENS defect were even further increased following inactivation of both *Sox8* alleles. During this study, $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ mice did not survive beyond 8 days of postnatal development. These mice were severely growth retarded and exhibited extended aganglionosis in large and small intestine. Action tremor was also repeatedly observed due to additional defects in CNS myelination (Stolt et al., 2004).

Sox8 is co-expressed with Sox10 during ENS development

During embryonic development of the ENS, Sox8 is co-expressed with Sox10. Both proteins are originally found in

the undifferentiated enteric neural crest precursors and are restricted later to the enteric glial lineage. Sox8 and Sox10 share very high sequence similarities in regions which are associated with such functions as transcriptional activation, DNA-binding, and subcellular localization (Kuhlbrodt et al., 1998a; Pfeifer et al., 2000; Schepers et al., 2000). Accordingly, biochemical data suggest that Sox8 and Sox10 have similar DNA binding and subcellular redistribution properties (Rehberg et al., 2002; Schepers et al., 2003; Schmidt et al., 2003; Stolt et al., 2004). Sox8 is also capable of activating Sox10 target genes (Stolt et al., 2004). The functional similarity between both proteins is further underscored by the recent finding that overexpression of Sox8 or Sox10 in the early chick neural tube was equally efficient in replacing Sox9 in its ability to induce ectopic neural crest cells (Cheung and Briscoe, 2003).

In general, co-expression causes functionally similar proteins such as Sox8 and Sox10 to perform redundant functions and to activate the same genetic programs. Such functional redundancy between Sox8 and Sox10 readily explains why loss of each additional *Sox8* allele in *Sox10* heterozygous mice leads to increasingly stronger ENS defects. However, it also makes the phenotypic differences between $Sox10^{+/lacZ}$ and $Sox8^{+/lacZ}$ mice difficult to conceptualize—especially given the fact that *Sox8* and *Sox10* are expressed at comparable levels from 12.5 to 18.5 dpc in the developing enteric nervous system. Why should heterozygous loss of *Sox10* lead to aganglionosis of the colon and loss of *Sox8* to no phenotype at all?

At the moment, it cannot be excluded that functional differences between Sox8 and Sox10 proteins so far escaped cell culture or biochemical studies. The two proteins might differ in their ability to interact with proteins that differentially modulate transcriptional capacity. Additionally, Sox8 might be less stable than Sox10 so that protein amounts for Sox8 are lower than for Sox10 despite similar expression levels. However, the conspicuous absence of survival and proliferation defects in enteric neural crest cells between 12.5 and 16.5 dpc argues for full compensation between both proteins during this phase of ENS development. Therefore, the differential requirement for Sox8 and Sox10 might not be in the enteric neural crest cells, but rather before in the vagal crest (see below).

ENS defects in mice with Sox10 and Sox8 deficiencies are not primarily due to defects in enteric glia

Both Sox10 and Sox8 are not only present during most phases of ENS development, they are already co-expressed in vagal neural crest before these cells enter the gut (Kapur, 1999; Sock et al., 2001). Thus, the ENS defect in Sox10 heterozygous mice could in principle be due to defective development of the vagal neural crest, the enteric neural crest or the enteric glial cell lineage. Additionally, several different processes could be affected as Sox10 has been shown to influence maintenance of pluripotency, survival, proliferation, specification and terminal differentiation under different conditions (Britsch et al., 2001; Kim et al., 2003; Paratore et al., 2001; Sonnenberg-Riethmacher et al., 2001; Stolt et al., 2002).

ENS development was already affected in the mouse mutants before enteric glia or their precursors appeared in significant numbers. Thus, it appears unlikely that the observed ENS phenotype is primarily caused by a glia-specific defect. This contrasts significantly with the situation in the sensory part of the peripheral nervous system, where in the absence of Sox10, glial cells fail to be specified, before sensory neurons degenerate later on (Britsch et al., 2001).

We did, however, observe a relative reduction of glial cells in $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ mice at 16.5 dpc. There were no B-FABP-positive glial precursors in the most distal colonized gut region. In the same region, neurogenesis was largely normal. This finding could be interpreted as a cell-intrinsic glial specification defect. As glial fate choice in neural crest cultures is dependent on community effects and requires a certain cell density (Paratore et al., 2001), glial specification could also be reduced because of decreased densities of enteric neural crest cells in this region. In the latter case, *Sox10* and *Sox8* mutations would only be indirectly accountable for the loss of glial cells. Interestingly, however, glial precursors were also reduced in the distal ileum of $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ mice which contained normal numbers of enteric neural crest cells at 16.5 dpc. This latter finding is compatible with a model in which Sox8 and Sox10 influence specification of enteric glia cell-intrinsically. Whether the observed reduction in enteric glia is transient or permanent needs to be established in future studies.

ENS defects in mice with Sox10 and Sox8 deficiencies are not primarily due to defects in the enteric neural crest

From earliest times onwards, colonization of the gut by enteric neural crest cells from the vagal region was significantly retarded in most embryos of a $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ or a $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ genotype, and in a minor fraction of $Sox10^{+/lacZ}$ embryos. Our data imply that the retardation observed at early times of ENS development is never compensated and might even correlate with the length of the aganglionic gut segment observed in postnatal animals. We also found no evidence in our compound mutants for significant colonization of the colon by neural crest stemming from the sacral region (Le Douarin and Kalchheim, 1999) indicating that the sacral crest in mammals might be similarly unable to replace missing vagal crest cells during ganglia formation in the colon as the chick sacral crest (Burns et al., 2000; Hearn and Newgreen, 2000).

Interestingly, even in those embryos where large segments of the midgut and hindgut remained uncolonized, colonized regions in moderate distance to the wavefront of

migrating enteric neural crest cells exhibited precursor cells and neurons in numbers comparable to the wildtype. In more general terms, this observation might point to the fact that continued forward migration of enteric neural crest cells is coupled to a mechanism that counts the density of enteric neural crest cells in the latest colonized areas so that migration is only continued once a certain cell density has been achieved.

If aganglionosis in *Sox10* heterozygous mice (with and without additional loss of *Sox8*) is caused by defective colonization, decreased proliferation or increased apoptosis of enteric neural crest cells might be responsible. Surprisingly, we did not find evidence for either model. Similar to endothelin receptor B (Shin et al., 1999), Sox10 might therefore not be essential for ENS development after 12.5 dpc.

Currently, we do not know whether loss of *Sox10* and *Sox8* alleles directly affects migration of enteric neural crest cells. However, we did not observe increased densities of enteric neural crest cells in the region closely behind the migratory wavefront in $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ or $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos. Also, migration was not halted at any defined region along the gut such as the caecum where changes of the gut environment impose particular strains on the migrating enteric neural crest cells. Still, migration is at least retarded so strongly that colon growth exceeds the migratory ability of the enteric crest cells in most $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ and $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos until the colon is no longer permissive for colonization.

ENS defects in mice with Sox10 and Sox8 deficiencies are due to defects in the vagal neural crest

Early studies in the chick already showed that partial ablation of the vagal crest led to a Hirschsprung-like aganglionosis of the colon (Yntema and Hammond, 1954). Therefore, the ENS defect in $Sox8^{+/lacZ}$, $Sox10^{+/lacZ}$ and $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ embryos could also be caused by an already decreased number of vagal crest cells. Analysis of these vagal neural crest cells at the time when they entered the gut revealed a strongly increased apoptosis in $Sox10^{+/lacZ}$ mice and an even higher rate in $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ mice. Rates of apoptosis were always significantly higher outside the gut, arguing that neural crest cells were much less susceptible to loss of *Sox10* and *Sox8* alleles once they had entered the gut. Previous studies had already shown that the vagal neural crest was affected much stronger by homozygous Sox10 mutations than the trunk neural crest and that complete aganglionosis in $Sox10^{dom/dom}$ embryos resulted from dramatic cell death in the vagal neural crest population (Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998). We show here that cell death is indeed so high in embryos with homozygous Sox10 mutation that in contrast to $Sox10^{+/lacZ}$ or $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ mice, vagal neural crest cells did not even reach and enter the gut. Our results favor a model in which the most important cause for the observed ENS defect is a reduced survival of vagal neural crest cells before they enter the gut, placing the ENS defect in our mice before 10 dpc and thus before the ENS defect in mice deficient for the endothelin receptor B (Shin et al., 1999). This, of course, does not rule out that Sox8 and Sox10 have additional consecutive roles in ENS development which might be less sensitive to dosage effects or masked by near complete redundancy between Sox8 and Sox10 at later times.

The phenotype of mice with combined *Sox8* and *Sox10* mutations must therefore be explained by the relationship of these two proteins in the vagal neural crest. Despite the increased rate of apoptosis in the $Sox10^{+/lacZ}$ neural crest, we detected 3-fold higher expression levels for *Sox10* than for the functionally redundant *Sox8*. Differential contribution to a common function in the vagal neural crest through such differences in amount could easily explain why heterozygous mutations of the major player Sox10, but not Sox8 as a minor contributor lead to gut aganglionosis and why Sox8 mutations invariably increase the severity of Sox10 mutations. Similar explanations have been invoked to explain oligodendrocyte and Sertoli cell phenotype in mice with combined Sox E gene mutations (Chaboissier et al., 2004; Stolt et al., 2004). We propose that such unequal contributions of related proteins to an essential function could be a common mode of action for genetic modifiers.

Whereas ENS defects in mice with Sox10 and Sox8 deficiencies are likely caused by a depletion of vagal neural crest before 10 dpc, defects observed upon inactivation of the endothelin-3 signaling pathway rather affect proliferation and migration of enteric crest cells between 10 and 12.5 dpc (Barlow et al., 2003; Kruger et al., 2003; Shin et al., 1999). Thus, despite similar phenotypic endpoints, the underlying cause of the ENS defect, the affected cell type, and the timing are different in Sox10-based and endothelin/endothelin receptor-based mouse models of Hirschsprung disease. Any attempt at treatment of human Hirschsprung disease would have to take this existence of multiple molecular ontogenies into account and determine the exact genetic cause first.

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