Neural crest development is regulated by the transcription factor Sox9

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Summary

The neural crest is a transient migratory population of stem cells derived from the dorsal neural folds at the border between neural and non-neural ectoderm. Following induction, prospective neural crest cells are segregated within the neuroepithelium and then delaminate from the neural tube and migrate into the periphery, where they generate multiple differentiated cell types. The intrinsic determinants that direct this process are not well defined. Group E Sox genes (*Sox8*, *Sox9* and *Sox10*) are expressed in the prospective neural crest and *Sox9* expression precedes expression of premigratory neural crest markers. Here, we show that group E Sox genes act at two distinct steps in neural crest differentiation. Forced expression of *Sox9* promotes neural-crest-like properties in neural tube progenitors at the expense of central nervous system neuronal differentiation. Subsequently, in migratory neural crest cells, SoxE gene expression biases cells towards glial cell and melanocyte fate, and away from neuronal lineages. Although SoxE genes are sufficient to initiate neural crest development they do not efficiently induce the delamination of ectopic neural crest cells from the neural tube consistent with the idea that this event is independently controlled. Together, these data identify a role for group E Sox genes in the initiation of neural crest development and later SoxE genes influence the differentiation pathway adopted by migrating neural crest cells.

Key words: Sox9, Neural crest, SoxE group transcription factors, Chick

Introduction

A common strategy exploited by many developing tissues is the establishment of a pool of self-renewing multipotent progenitors that generate the differentiated cell types that form the mature tissue. These progenitors, or stem cells, are usually segregated spatially and functionally from surrounding tissues early in embryogenesis and initiate a distinct transcriptional programme in response to extrinsic cues. Although studies have begun to identify the signalling molecules and intrinsic determinants that control these processes, there is in most cases only a partial understanding of the mechanisms deployed. An example of this is the neural crest – a transient migratory population of stem cells that originates from cells in the dorsal neural folds at the border of the neural plate and epidermal ectoderm. Following induction, prospective neural crest cells delaminate from the neural tube and take characteristic migration pathways into the periphery, where they differentiate into multiple cell types, notably neurons and glia of the peripheral nervous system, as well as pigment-producing melanocytes of the skin (Le Douarin and Kalcheim, 1999; Nieto, 2001; Knecht and Bronner-Fraser, 2002).

The signalling events that instruct neural crest development have received much attention. Inductive interactions between the epidermal ectoderm and neural plate are required for induction and several candidate signals have been proposed to mediate this event. Most consideration has been given to the BMP and Wnt groups of secreted factors. Members of both families of molecules are expressed in the relevant tissues at appropriate times during development and there is evidence that each family of proteins is necessary and sufficient to induce neural crest (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Liem et al., 1995; Liem et al., 1997; Ikeya et al., 1997; Selleck et al., 1998; Garcìa-Castro et al., 2002). Although these studies suggest apparently contradictory models, it is possible that both sets of signals are involved in neural crest induction, perhaps at different stages of development or in different capacities (Aybar and Mayor, 2002). Additional signals such as fibroblast-derived growth factors (FGFs) and retinoic acid have also been implicated in the induction and differentiation of neural crest and their role also remains to be clarified (Mayor et al., 1997; Villanueva et al., 2002).

Within prospective neural crest cells, the transcriptional programme that is initiated in response to the inductive signal is not clearly defined. The dorsal neural tube expresses several transcription factors in response to neural-crest-inducing signals. Examples include Pax3, Msx1-3 and Zic1-3. Although these proteins have been implicated in neural crest induction, they are also expressed in neural progenitors that generate dorsal interneurons and so are unlikely to be involved solely in neural crest induction (Epstein et al., 1991; Liem et al., 1995; Houzelstein et al., 1997; Nakata et al., 1997; Nakata et al., 1998). Several transcription factors have been identified that are restricted to developing neural crest. These include AP2, Id2, FoxD3 and Slug, but the epistatic relationships between these proteins and the relative contribution of each to neural

crest specification remain unclear. Loss of AP2 results in defects in neural crest development (Schorle et al., 1996; Zhang et al., 1996) and the forced expression of AP2 in frog embryos is sufficient to induce neural crest differentiation (Luo et al., 2003). However, AP2 is initially expressed throughout the ectoderm, suggesting that other factors must be involved in restricting neural crest induction to the appropriate region (Luo et al., 2002). Id2, a basic helix-loop-helix transcription factor, is sufficient to induce neural crest characteristics in the chick neural tube (Martinsen and Bronner-Fraser, 1998), but Id2 is expressed only in cranial regions and mice lacking *Id2* have no reported neural crest defects (Yokotak et al., 1999). The zinc-finger transcription factor Slug and the forkhead class transcription factor FoxD3 are the strongest candidates for general neural-crest-specifying factors. Both are expressed transiently in neural crest cells prior to delamination (Nieto et al., 1994; Dottori et al., 2001; Kos et al., 2001). However, the absence of Slug in mouse does not affect delamination (Jiang et al., 1998) and the effect of ectopic expression of Slug is limited to increasing the amount of neural crest specification in cranial regions of the neural tube (del Barrio and Nieto, 2002). Moreover, although the forced expression of FoxD3 induces some aspects of neural crest differentiation in the ventral neural tube, it is not sufficient to induce cells that exhibit all the characteristics of neural crest (Dottori et al., 2001; Kos et al., 2001). Together, these studies raise the possibility that other transcription factors are involved in the specification of prospective neural crest cells.

Members of the Sox gene family of high-mobility-group (HMG) domain containing transcription factors are candidates for playing a role in neural crest specification. Sox proteins are involved in several processes during embryogenesis. Based on the amino acid sequence of the HMG domain, Sox proteins can be divided into ten sub groups (Bowles et al., 2000). Subgroup E consists of three members (Sox8, Sox9 and Sox10) that are expressed in several developing tissues, including the neural crest. Mice lacking Sox8 develop to adulthood without severe defects (Sock et al., 2001). By contrast, loss of function analyses have identified roles for Sox9 and Sox10 in neural crest development. In frog embryos, morpholino-mediated depletion of Sox9 results in loss of neural crest progenitors (Spokony et al., 2002). It is not clear whether this reflects a requirement for Sox9 in neural-crest cells or a role for Sox9 in controlling neural-crest-inducing signals, and it remains to be established whether Sox9 is sufficient to initiate neural crest development. Loss of function studies indicate that Sox10 has a role in later aspects of neural crest development. In mice and zebrafish lacking Sox10, the early specification of neural crest is unaffected but the later differentiation of peripheral glial cells and melanocytes is disrupted (Britsch et al., 2001; Dutton et al., 2001). Moreover, recent studies (Kim et al., 2003; Paratore et al., 2002) have proposed a role for Sox10 in maintaining the multipotency of neural crest stem cells as well as directing differentiating cells to non-neuronal fates. Together, these studies have focused attention on SoxE genes in neural crest development, but the role these genes play in the early events in neural crest differentiation remain to be resolved. Because functional redundancy between SoxE family members might limit the phenotypes observed in the loss of function analyses, we have taken a gain of function approach to examine the role these genes play in neural crest induction.

We demonstrate that SoxE genes are expressed in premigratory neural crest and that Sox9 is an early marker of prospective neural crest, preceding markers of migratory neural crest. Forced expression of Sox9 or other group-E Sox genes in the neural tube induces ectopic neural crest differentiation at the expense of central nervous system (CNS) neuronal generation. Strikingly, although Sox9 induces many neural crest markers along the entire dorsal-ventral axis of the neural tube, efficient emigration of ectopic neural crest is restricted to the most dorsal regions. Ventral to this, delaminating cells are observed only infrequently. This supports a model in which the induction and delamination of neural crest are independent events. Consistent with this, RhoB, which has been implicated in promoting delamination, is not induced by Sox9, raising the possibility that delamination is initiated by the upregulation of a subset of factors including RhoB. In the periphery, SoxE-transfected neural crest cells migrate along typical neural crest routes and display characteristics of glial and melanocyte neural crest derivatives but are excluded from neuronal lineages, indicating that continued expression of SoxE genes biases differentiation to certain neural crest lineages. Together, our findings indicate that SoxE genes act at two stages of neural crest differentiation – first as cell intrinsic determinants of neural crest, initiating neural crest development and segregating this lineage from the neuroepithelium, and subsequently directing differentiation decisions in the periphery, biasing neural crest cells to glial cell and melanocyte lineages and away from neuronal fates.

Materials and methods

In situ hybridization and immunohistochemistry

Fertilized chick eggs were obtained from Winter Egg Farm (Royston, UK) and incubated in a humidified incubator at 38°C. Embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951). Embryos were fixed for an hour at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), cryoprotected with 30% sucrose in PB and cryosectioned. Frozen section in situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993), using probes for chick *Sox8*, *Sox9*, *Sox10* (Cheng et al., 2000; Cheng et al., 2001), chick *Slug* (Nieto et al., 1994), *BMP4*, *BMP7* (a gift from T. Momose), *Cad6B*, *RhoB* (Liu and Jessell, 1998), *Cad7* (a gift from Y. Wakamatsu), *Wnt1*, *Wnt3a* (a gift from N. Itasaki), *FoxD3* and *CyclinD1* chick expressed sequence tag (EST) clones. Immunohistochemical localization of proteins on sections and neural plate explants in collagen gels were performed as described (Yamada et al., 1993; Briscoe et al., 2001). Whole-mount immunofluorescence was carried out as described (Davis et al., 1991). Antibodies against the following proteins were used: green fluorescent protein (GFP) (Molecular Probes), Sox9 (da Silva et al., 1996), Slug (Liu and Jessell, 1998), Laminin (Sigma), Pax2 (Covance), TuJ1 (Covance), HNK-1 (Becton Dickinson), Lbx1 (Müller et al., 2002) and pan-Nkx6 (a gift from H. Edlund), Pax6, Pax7, MNR2 (Persson et al., 2002), Islet1/2 (Vallstedt et al., 2001), P0 (Bhattachyaryya et al., 1991) and BrdU (bromodeoxyuridine) (George-Weinstein et al., 1993). Images were collected on a Zeiss LSM510 or Leica TCS SP2 confocal microscope.

Chick in ovo electroporation and BrdU labelling

Chick *Sox9* cDNA (Kamachi et al., 1999), chick *Sox8* cDNA (Cheng et al., 2001), chick *Sox10* cDNA (Cheng et al., 2000) and *Wnt3a* (a gift from N. Itasaki) were inserted upstream of an internal ribosomal entry site (IRES) and nuclear localization sequence (nls) tagged GFP in pCAGGS expression vector (Niwa et al., 1991). *Sox2* cDNA was

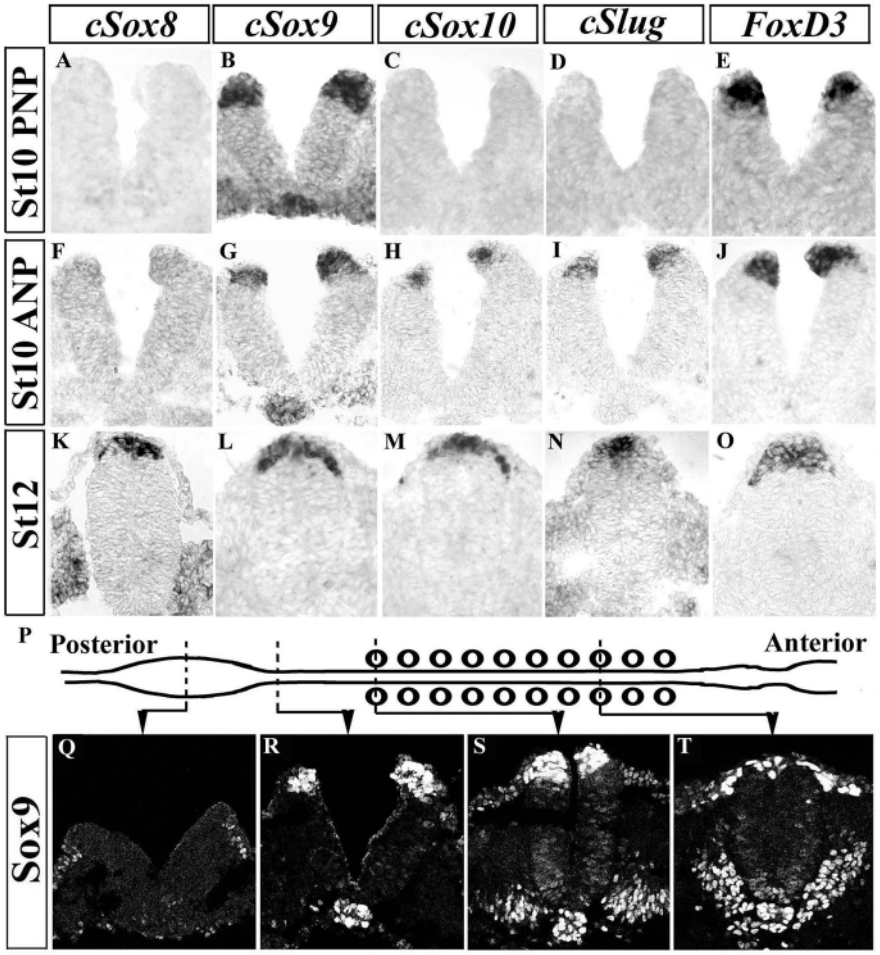


Fig. 1. Sox9 is expressed in prospective neural crest cells. Expression of the indicated genes in sections of the posterior and anterior open neural plate (PNP and ANP, respectively), and neural tube of HH stage 10 and stage 12 chick embryos. In posterior neural plate (B), *Sox9* expression is restricted to the dorsal tips of the neural fold region, overlapping with *FoxD3* (E), and precedes expression of *Sox8* (A), *Sox10* (C) and *Slug* (D). Anterior to this, expression of *Sox10* (H) and *Slug* (I) are also found in a similar domain to *Sox9* (G) and *FoxD3* (J), but *Sox8* (F) is still not detected in this domain. After neural tube closure, *Sox8* (K), *Sox9* (L), *Sox10* (M), *Slug* (N) and *FoxD3* (O) are expressed in the dorsal midline, where premigratory neural crest is located. Stage 10 images are adjacent sections from the open neural plate region of a HH stage 10 embryo. Stage 12 sections are from the prospective forelimb level of HH stage 12 embryos. (P) Schematic diagram of HH stage 10 chick embryo showing anterior and posterior levels of sections for the images in Q-T. (Q-T) Confocal images of *Sox9* expression in HH stage 10 chick embryo from posterior to anterior levels. (Q) Sox9 is not detected in the most posterior regions. Anterior to this, Sox9 is upregulated in the dorsal tips of the closing neural folds (R,S). *Sox9* expression is detected in the premigratory crest region at the more anterior region of closed neural tube (T). Expression of *Sox9* is also seen at high level in the notochord (R-T).

cloned into the pCS2+ expression vector (a gift from E. Remboutsika). Chick embryos were electroporated with DNA at 2.5 μg μl^{-1}, for co-transfections, pCAGGS-IRES-nls-GFP was used at 500 ng μl^{-1}. Briefly, plasmid DNA was injected into the lumen of HH stage 10-11 neural tubes, electrodes placed either side of the neural tube and electroporation carried out using a BTX electroporator delivering five 50 millisecond pulses of 30 V (Briscoe et al., 2001). Transfected embryos were allowed to develop to the specified stages then dissected, fixed and processed for immunohistochemistry. Transfection of pCAGGS-IRES-nls-GFP alone does not affect expression of neural markers or neural crest markers (data not shown). For BrdU labelling, 100 μl of 200 ng μl^{-1} BrdU (Roche Biochemicals) was applied on top of the transfected embryos in ovo 1 hour before harvesting.

Neural plate explants

For explant culture of electroporated neural tissue, HH stage 10 embryos were electroporated with pCAGGS-Sox9-IRES-nls-GFP or pCAGGS-IRES-nls-GFP as a control and incubated in ovo for 1-2 hours before isolating the neural explants (Yamada et al., 1993; Briscoe et al., 2001). Neural explants were cultured in collagen matrix (Vitrogen) with F12 medium containing penicillin/streptomycin and Mito$^+$ Serum Extender (Collaborative Biomedical Products) for 48 hours before assaying GFP and HNK-1 expression (Liem et al., 1995). For transplantations, electroporated [i] (intermediate neural tube) regions were placed between the neural tube and somite at the forelimb level of HH stage 12-14 chick embryos. Transplanted embryos were incubated for 72 hours before processing for immunohistochemistry.

Results

Group E Sox genes are expressed in premigratory and early migratory neural crest

To examine the function of group E Sox genes (*Sox8*, *Sox9* and *Sox10*) in early neural crest specification, we first determined their spatial expression patterns and compared them to factors proposed to be involved in neural crest induction. In prospective trunk regions of the open neural plate of stage 10 chick embryos, expression of chick *Sox9* was detected in cells at the dorsal tips of the neural folds (Fig. 1B,G). In the posterior open neural plate, *Sox9* expression overlaps with that of *FoxD3*; anterior to this, chick *Slug*, *Cadherin6B* (*Cad6B*), *AP2*, *RhoB*, *BMP4*, *Wnt1* and *Wnt3a* are also detected in a similar domain (Fig. 1B,E,I and data not shown) (Liem et al., 1995; Hollyday et al., 1995; Kos et al., 2001; Nakagawa and Takeichi, 1998; Liu and Jessell, 1998; Luo et al., 2002). By stage 12, after neural plate closure, the expression of *Sox9* continued in cells in the dorsal midline of the neural tube (Fig. 1L), overlapping with cells expressing *Slug*, *FoxD3*, *Cad6B*, *AP2*, *RhoB*, *BMP4*, *BMP7*, *Wnt1* and Wnt3a (Fig. 1N,O and data not shown). Expression of chick *Sox8* and *Sox10* (Fig. 1K,M) were also induced in a similar domain. Thus, the domain of *Sox9* expression in dorsal trunk neural tube corresponds to the region containing premigratory neural crest (Fig. 1L). By stage 12, emigration of neural crest cells had commenced and expression of *Sox10*, *FoxD3*, *AP2* and *RhoB* (Fig. 1M,O and data not

shown) was detected in migrating neural crest cells adjacent to the neural tube. The expression of *Sox8*, *Sox9* and *Sox10* persisted in the dorsal midline until stage 18 and was then downregulated (data not shown). Regions of expression of group E Sox genes are also evident outside the neural crest and have been described previously (Bell et al., 2000; Zhao et al., 1997; Kuhlbrodt et al., 1998; Cheng et al., 2000; Cheng et al., 2001).

To define the pattern of Sox9 expression in more detail, we took advantage of an antiserum raised against Sox9 (da Silva et al., 1996) and examined Sox9 production along the anterior-posterior axis of HH stage 10 chick embryos (Fig. 1P). At the level of Henson's node and posterior open neural plate, Sox9 production was detected in non-neural ectoderm (Fig. 1Q). Anterior to this in the closing neural plate, Sox9 was robustly induced in the tips of the neural fold (Fig. 1R,S). At more rostral levels, after neural tube closure, Sox9 production was detected in the dorsal midline of the neural tube (Fig. 1T). Commencing at stage 14-15, Sox9 was induced in ventral-dorsal gradient in the neural tube, but this neural production of Sox9 was initially at low levels and did not peak until approximately 48 hours later, at stage 18-20 (data not shown). These data are consistent with the expression of *Sox9* RNA. Together, the data indicate that *Sox9* is expressed in premigratory neural crest cells. Moreover, *Sox9* expression is induced in naive neural plate explants by the neural-crest-inducing signal BMP4 (M.C. and J.B., unpublished). Sox9 therefore represents an early marker of prospective neural crest cells.

Sox9 induces neural crest differentiation in neural cells

The expression profile of *Sox9* raised the possibility that Sox9 is involved in neural crest specification. To test this idea, *Sox9* was ectopically expressed in the neural tube of stage 10-11 chick embryos by in ovo electroporation. A bicistronic vector was used that encoded Sox9 and nuclear targeted GFP (a marker used to identify transfected cells), resulting in the unilateral mosaic expression of *Sox9* in the neural tube (Briscoe et al., 2001). Our analysis focuses on trunk regions of the neural tube between the forelimbs and hindlimbs. We first examined the expression of the migratory-neural-crest marker HNK-1 (Bronner-Fraser, 1986). Embryos transfected with a control GFP vector did not induce ectopic HNK-1 at any of the time points examined (data not shown). By contrast, extensive, robust ectopic induction of HNK-1 production was detected in neural progenitor cells of embryos electroporated with *Sox9* 12-48 hours after electroporation (Fig. 2D-I and data not shown, in 30/30 embryos). The activity of Sox9 appeared to be cell autonomous – only transfected cells induced HNK-1 and adjacent untransfected cells lacked HNK-1 expression (Fig. 2F,I). Moreover, transfected Sox9 also induced other markers of neural crest including *Slug*, *Cad6B* and *Cad7* (see Fig. 4). Transfection of vectors directing expression of Sox8 and Sox10 also induced HNK-1 expression with similar kinetics (data not shown, 15/15 embryos). Conversely, the transfection of a Sox gene from a different subgroup (*Sox2*, a group B Sox gene) did not induce ectopic HNK-1 (data not shown, 6/6 embryos). Together, these data suggest that *Sox9* and other group E Sox genes induce neural crest differentiation in cells normally expected to generate CNS neurons.

To test directly whether Sox9 is sufficient to induce neural crest differentiation in neural cells not normally expected to generate neural crest, we examined the influence of *Sox9* expression on neural progenitor cells grown in vitro. Stage 10 chick embryos were electroporated in ovo with *Sox9* or control vector and intermediate [i] neural explants from electroporated embryos were isolated 1-2 hours later and grown in vitro for 24-48 hours. Explants transfected with the control vector (n=8) did not express significant levels of *HNK-1* and cells did not emigrate from the explant (Fig. 2N-Q). By contrast, high levels of *HNK-1* expression were evident in explants transfected with *Sox9* (n=7) (Fig. 2R-T). Moreover, migratory cells were found emigrating from these explants (Fig. 2R,U), and the migratory cells expressed *HNK-1* and displayed mesenchymal morphology consistent with these cells being neural crest (Fig. 2R-U and data not shown). The effect of *Sox9* expression was cell autonomous – only GFP-positive cells were found migrating away from transfected explants (Fig. 2T). These data indicate that Sox9 is sufficient to induce neural crest differentiation in a cell-autonomous manner.

The finding that *Sox9* expression induced migration of cells in vitro prompted us to examine in ovo electroporated embryos in more detail. 24-48 hours post-transfection (hpt), a marked increase in the number of HNK-1$^+$ neural crest cells was observed migrating away from the transfected side of the neural tube (Fig. 2J,K and data not shown, n=6). These cells appeared to originate dorsally (Fig. 2G-I). Consequently, by 24 hpt, the most dorsal regions of the neural tubes were frequently depleted of cells compared with the untransfected side of the embryo (Fig. 2G-I). Consistent with this, there was an increase in basement membrane disassembly on the transfected side of embryos (Fig. 2L, data not shown). Ventral to this, there were *Sox9*-transfected cells expressing *HNK-1* that migrated laterally through the pial surface (Fig. 2I,L,M). It was, however, noticeable that the delamination of *Sox9*-expressing cells in these intermediate and ventral regions of the neural tube was limited to a minority of the transfected population (Fig. 2L,M). Most *Sox9*-expressing cells at these dorsal-ventral positions remained within the neural tube and displayed the pseudostratified epithelial morphology characteristic of the neural tube despite expressing *HNK-1* (Fig. 2D-I,M). Together, these data indicate that *Sox9* can induce the differentiation of neural crest cells and, in some circumstances, these cells emigrate from the neural tube. However, cells only delaminate efficiently from dorsal regions of the neural tube. More ventrally, most cells remain within the neuroepithelium, suggesting that the initiation of neural crest delamination is constrained to the dorsal neural tube.

Sox9 induction of neural crest does not require BMP or Wnt signals

The ability of *Sox9* to induce markers and behaviour of neural crest cells prompted us to examine the pathway of neural crest induction. The secreted factors BMP4, BMP7, Wnt1 and Wnt3a are expressed in dorsal regions of the neural tube and have been implicated in inducing neural crest differentiation (Liem et al., 1995; Ikeya et al., 1997; LaBonne and Bronner-Fraser, 1998; Garcìa-Castro et al., 2002). Forced expression of *Sox9* did not induce expression of *BMP4* (Fig. 3A-C, n=6), *BMP7* (Fig. 3D-F, n=6) or *Wnt1* (Fig. 3G-I, n=6) at any time point examined. Indeed, in transfected embryos, endogenous

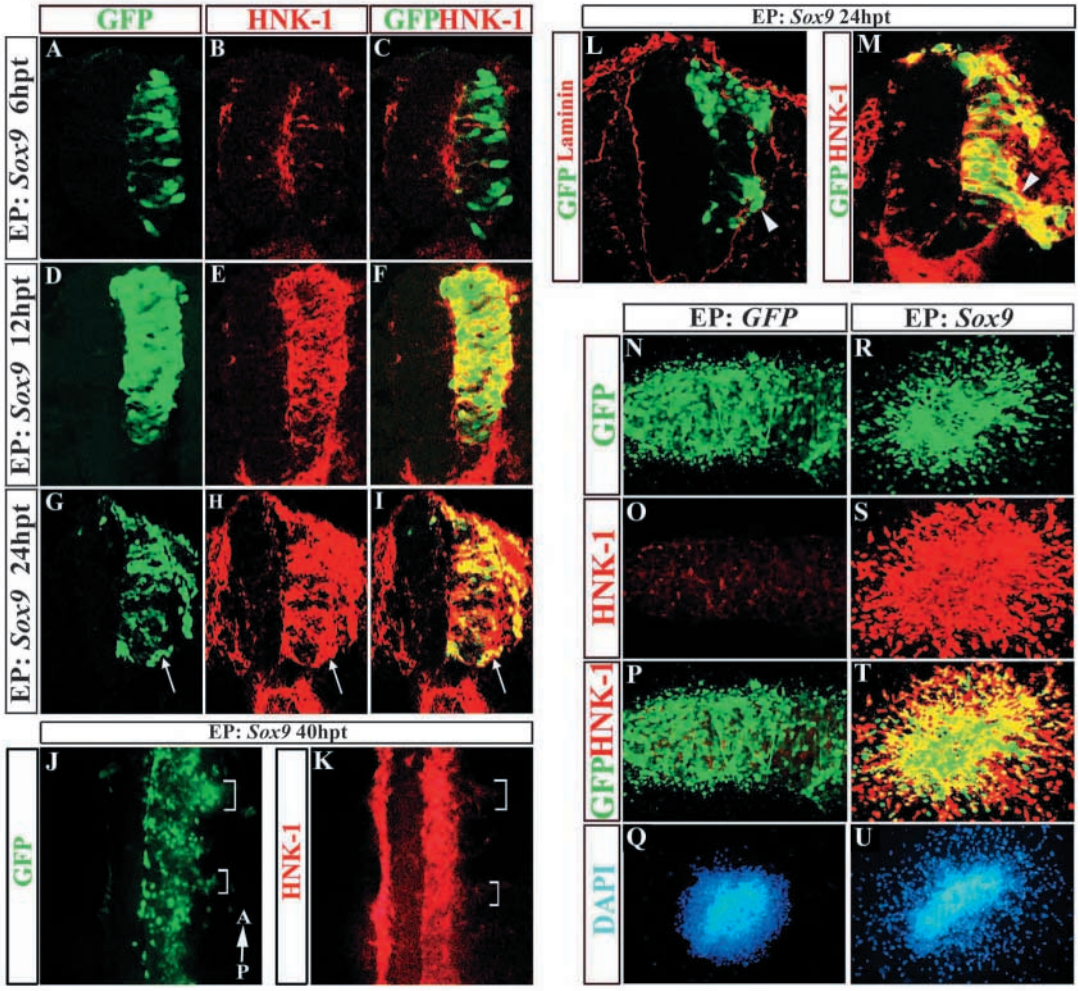


Fig. 2. Sox9 induces *HNK-1* expression and neural crest differentiation. (A-I) Neural tubes electroporated with *Sox9*, 6 (A-C), 12 (D-F) and 24 (G-I) hours post transfection (hpt) analysed for *HNK-1* expression. High level expression of the transfected construct was detected within 6 hpt (A-C) and robust ectopic induction of *HNK-1* was detected by 12 hpt (D-F) and continued at 24 hpt (G-I) following *Sox9* electroporation (EP). Confocal images indicate that the effect of Sox9 on *HNK-1* expression is cell autonomous (C,F,I and data not shown). (G-I) An increase in the number of neural crest cells delaminating dorsally was evident and several transfected cells were observed delaminating from the neural tube in the intermediate and ventral region of the neural tube at 24 hpt (white arrows). (J) Dorsal view of the trunk neural tube electroporated with *Sox9* at 40 hpt and assayed for *HNK-1* expression by whole-mount immunofluorescence (K). *Sox9*-expressing cells are observed in the neural tube and in the delaminating neural crest cells (J, white brackets). An increase in the amount of delaminating *HNK-1*-expressing neural crest cells is detected on the transfected side of the embryo (K, white brackets). (L) *Sox9*-expressing cells are observed delaminating from the pial surface of the dorsal and intermediate region (white arrowhead) of the neural tube, corresponding to regions where laminin production is lost. (M) *Sox9*-expressing cells with ectopic *HNK-1* expression are observed delaminating from the ventral neural tube (white arrowhead). (N-U) Induction of neural crest cells by Sox9 in neural plate explants. Vectors encoding either Sox9 and GFP or GFP alone were electroporated into the open neural plate region of the HH stage 10 chick embryos, [i] regions dissected and cultured for 48 hours in vitro before examining *HNK-1* expression. (N-P) Explants of [i] regions transfected with GFP alone do not induce *HNK-1* expression (O,P) and the DAPI image indicates cells remain confined to the explant (Q). By contrast, ectopic *HNK-1* expression is detected in [i] explants transfected with *Sox9* and many cells emigrate from the explants (R-U). A, anterior; P, posterior.

expression of these signalling molecules was downregulated in the roof plate by 12 hpt (Fig. 3B,E,H); this is likely to be due to the increased delamination and consequent loss of roof plate cells from this region. Furthermore, transfection of a construct directing the expression of the BMP antagonist Noggin together with Sox9 did not inhibit induction of ectopic neural crest (M.C. and J.B., unpublished). Moreover, Sox9 did not induce expression of *Pax3*, *Pax7* or other genes characteristic of generic dorsal neural tube identity that have previously been characterized as responding to dorsalizing signals (data not shown) (Liem et al., 1995; Liem et al., 1997).

By contrast, transfection of Sox9 induced *Wnt3a* at 6-12 hpt (Fig. 3J,K, *n*=8). To test whether the expression of *Wnt3a* might explain neural crest induction by Sox9, we examined the effect of ectopic *Wnt3a* expression in the neural tube. Consistent with previous studies, expression of *Wnt3a* (Fig. 3M, *n*=3) increased proliferation of neural progenitors (Fig. 3P), induced *CyclinD1* expression in ventral neural tube regions (Fig. 3Q) and inhibited neuronal differentiation (Fig. 3R) (Megason and McMahon, 2002). However, no ectopic *Sox9* or *HNK-1* expression or neural crest induction was detected in any region of transfected neural tubes (Fig. 3M-O and data not shown). These data indicate that Wnt3a is not sufficient to induce neural crest production in the neural tube and indicate that Wnt3a is downstream of Sox9 in the pathway of neural crest induction. Together, these data suggest that Sox9 does not act via the induction of *BMP4*, *BMP7*, *Wnt1* or *Wnt3a* and are consistent with the cell autonomous induction of neural crest in *Sox9*-transfected embryos and explants.

Sox9 induces a subset of early neural crest markers

We next examined factors that have been implicated in the

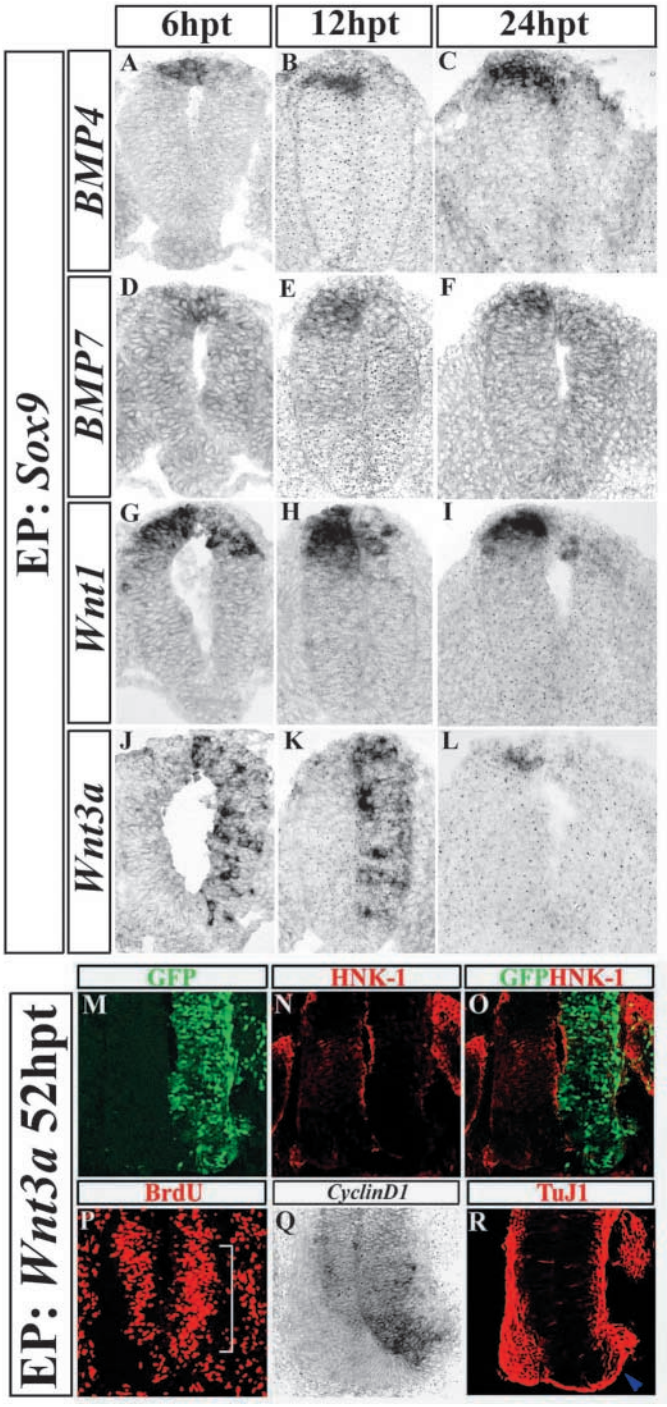


Fig. 3. Neural crest cell induction by Sox9 is not dependent on BMP or Wnt signals. Panels show embryos electroporated with *Sox9* (A-L) or *Wnt3a* (M-R) and analysed at the indicated times after transfection; images are oriented with the transfected side of the neural tube to the right. Induction of *BMP4* (A-C), *BMP7* (D-F) and *Wnt1* (G-I) was not detected at any time points examined. Expression of *BMP4* (A-C), *BMP7* (D-F) and *Wnt1* (G-I) was reduced in the dorsal neural tube, which might be a consequence of a change in fate of these cells from neural progenitor to neural crest. (J-L) By contrast, *Wnt3a* is induced throughout the dorsal/ventral region of the neural tube at 6 hpt (J) and 12 hpt (K) but returns to basal levels by 24 hpt (L). (M-O) Ectopic expression of *Wnt3a* does not induce *HNK-1* expression. (P) *Wnt3a* transfection increases BrdU incorporation in the ventral neural tube (white bracket). Moreover, *CyclinD1* (Q) expression is induced ventrally and expression of the neuronal marker *TuJ1* (R) is reduced. The increased proliferation results in deformities on the transfected side of the embryo (blue arrowhead).

cell autonomous neural crest differentiation programme. Premigratory crest cells express *Slug*, *FoxD3* and *Cad6B* (Nieto et al., 1994; Luo et al., 2002; Kos et al., 2001; Nakagawa and Takeichi, 1998), whereas migratory crest cells express *Sox10*, *Cadherin7* (*Cad7*) and *HNK-1* (Cheng et al., 2000; Nakagawa and Takeichi, 1998; Bronner-Fraser, 1986). We first focused on intermediate and ventral regions of the neural tube, where *Sox9* did not initiate efficient delamination. Expression of *Sox9* in these regions induced the expression of neural crest markers. Similar to the endogenous expression profile, ectopic *Slug* and *Cad6B* (Fig. 4A-F, *n*=16) were transient, induced at 6 hpt and downregulated by 12 hpt (Fig. 4B,E, *n*=11). The induction of migratory neural crest markers was slower but sustained. Robust *Cad7* expression was not seen until 12 hpt (Fig. 4H, *n*=8) and ectopic expression of *Sox10* was first evident 12-24 hpt (Fig. 4N,O; 10/12 embryos). *FoxD3*, which is expressed in both premigratory and migrating neural crest, was induced only at later times, 12-24 hpt (Fig. 4K,L; 9/12 embryos), raising the possibility that the early and late phases of *FoxD3* expression are independently controlled.

We next turned our attention to the dorsal region of the neural tube where ectopic *Sox9* induces a marked increase in the numbers of neural crest cells delaminating. In this domain, as ventrally, expression of premigratory neural crest markers *Slug* and *Cad6B* (Fig. 4A,D) was detected at 6 hpt. By 12 hpt, a decrease in the expression of these markers was evident (Fig. 4B,E). At later time points, 12-24 hpt, the migratory markers *Cad7*, *FoxD3* and *Sox10* (Fig. 4H,I,K,L,N,O) were observed in or adjacent to the dorsal neural tube. The rapid downregulation of premigratory markers on the transfected side of the neural tube contrasts with untransfected regions, where expression of premigratory markers continues in the dorsal midline at these stages (Fig. 4B,E). Taken together, these data suggest that *Sox9* is sufficient to induce expression of neural crest markers in the neural tube. The inhibition at 12 hpt of premigratory markers in dorsal regions suggests that expression of *Sox9* accelerates the premigratory to migratory transition of prospective neural crest cells, synchronizing the differentiation of all prospective neural crest cells. By 12 hpt, therefore, all cells in the dorsal neural tube have transited through the premigratory stage and matured to more differentiated stages, leaving none to continue expressing premigratory markers.

In contrast to the expression of the neural crest markers examined above, *Sox9* failed to induce *RhoB* at any time point examined (Fig. 4P-R, *n*=18). *RhoB* is expressed in premigratory neural crest and has been implicated in promoting the delamination of neural crest cells (Liu and Jessell, 1998). Although the expression of *RhoB* was downregulated by 12 hpt in the dorsal domain of progenitors (Fig. 4Q) in a similar manner to the other premigratory makers examined (Fig. 4B,E), ectopic *RhoB* expression was never observed at ventral or intermediate positions (Fig. 4P-R). This indicates that *Sox9* is not sufficient to induce *RhoB*. The lack of *RhoB* induction together with the finding that *Cad7* (Fig. 4H,I) and *HNK-1*

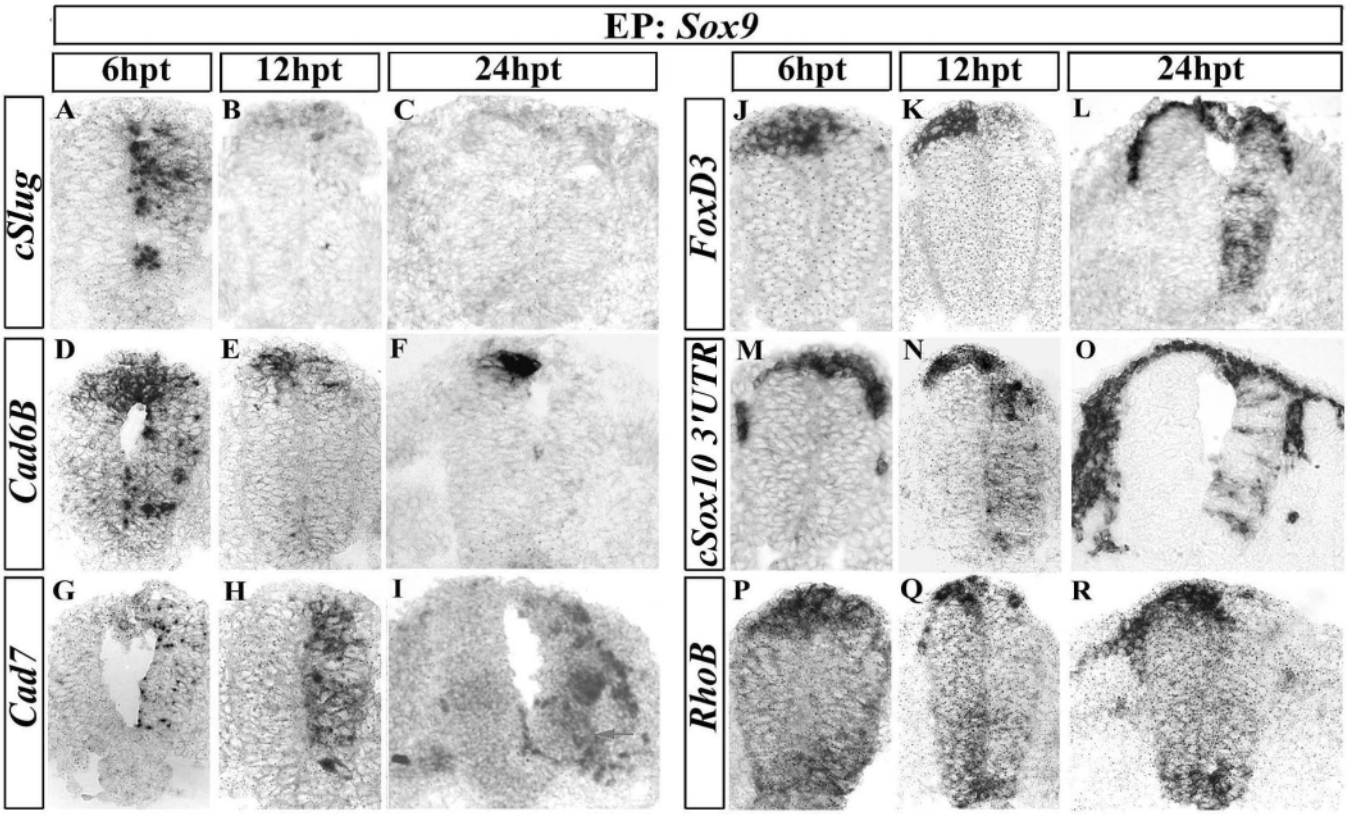


Fig. 4. Sox9 induces neural crest markers but not RhoB. All panels show embryos electroporated with *Sox9* and analysed at the indicated times after transfection; images are oriented with the transfected side of the neural tube to the right. *Slug* (A-C) and *Cad6B* (D-F) are induced transiently by Sox9; upregulation can be detected at 6 hpt (A,D) but expression levels have returned to basal by 12 hpt and 24 hpt (B,C,E,F). (G-I) Ectopic *Cad7* expression is observed at all time points and ectopic expression of *Cad7* is observed in cells migrating away from the neural tube (I, red arrow). (J-L) Ectopic *FoxD3* expression is also detected in transfected neural tube but not until 24 hpt (L). *Sox10* expression is normal at 6 hpt (M) but ectopic induction can be detected at 12 hpt (N) and continues at 24 hpt (O). By contrast, *RhoB* is not induced in any time point examined (P-R) and reduction of endogenous expression is observed at 6 hpt (P), 12 hpt (Q) and 24 hpt (R).

(Fig. 2D-I), which are normally restricted to migratory neural crest cells (Nakagawa and Takeichi, 1998; Bronner-Fraser, 1986), are expressed within the neural tube in cells with a pseudostratified epithelial morphology support the idea that, although *Sox9* initiates the transcriptional programme of neural crest development, it is not sufficient to promote the delamination of neural crest cells. The efficient emigration of neural crest cells is constrained to dorsal regions of the neural tube and the expression of *RhoB* appears to define the region that contains cells competent to delaminate.

Sox9 suppresses the normal differentiation programme of neural progenitor cells

The induction of neural crest differentiation in *Sox9*-transfected neural progenitor cells led us to examine whether this was at the expense of the normal differentiation programme of neural cells. To address this, we assayed the expression of progenitor and neuronal subtype markers in the neural tube of *Sox9*-electroporated embryos. Ectopic *Sox9* repressed the expression of the neural progenitor markers *Pax7* (Fig. 5A-C, *n*=7), *Pax6* (Fig. 5D-F, *n*=6), *Nkx6* (Fig. 5G-I, *n*=6), *Irx3*, *Olig2* and *Nkx2.2* (data not shown) (Briscoe et al., 2000; Novitch et al., 2001). The repressive activity of *Sox9* was cell autonomous – only transfected cells demonstrated a change in expression profile of the progenitor makers. Consistent with these data, *Sox9* blocked the generation of classes of spinal neurons including *Lbx1* (Fig. 5J-L, *n*=8) and *Pax2* (Fig. 5M-O, *n*=6) expressing interneurons (Müller et al., 2002; Burrill et al., 1997) and $MNR2^{+}/HB9^{+}$ motor neurons (Fig. 5P-R, *n*=6) (Tanabe et al., 1998). These neurons are each generated at different dorsoventral positions and, within each domain, cells that did not express *Sox9* generated neuronal subtypes in a position-appropriate manner, indicating that the repression of neuronal generation is also cell autonomous. We conclude that the expression of *Sox9* leads to cell autonomous suppression in neural progenitor identity and neuronal differentiation.

Expression of *Sox9* in migrating neural crest biases cells to glial and melanocyte fates

The delamination and lateral migration of *Sox9*-expressing cells led us to examine whether these cells differentiate into neural crest derivatives. At trunk levels, the predominant differentiated neural crest cell types are neuronal, glial and pigment cells (Le Douarin and Kalcheim, 1999). To determine the effect of the expression of *Sox9* on neural-crest differentiation, transfected embryos were examined 24-72 hours after electroporation. In control embryos electroporated

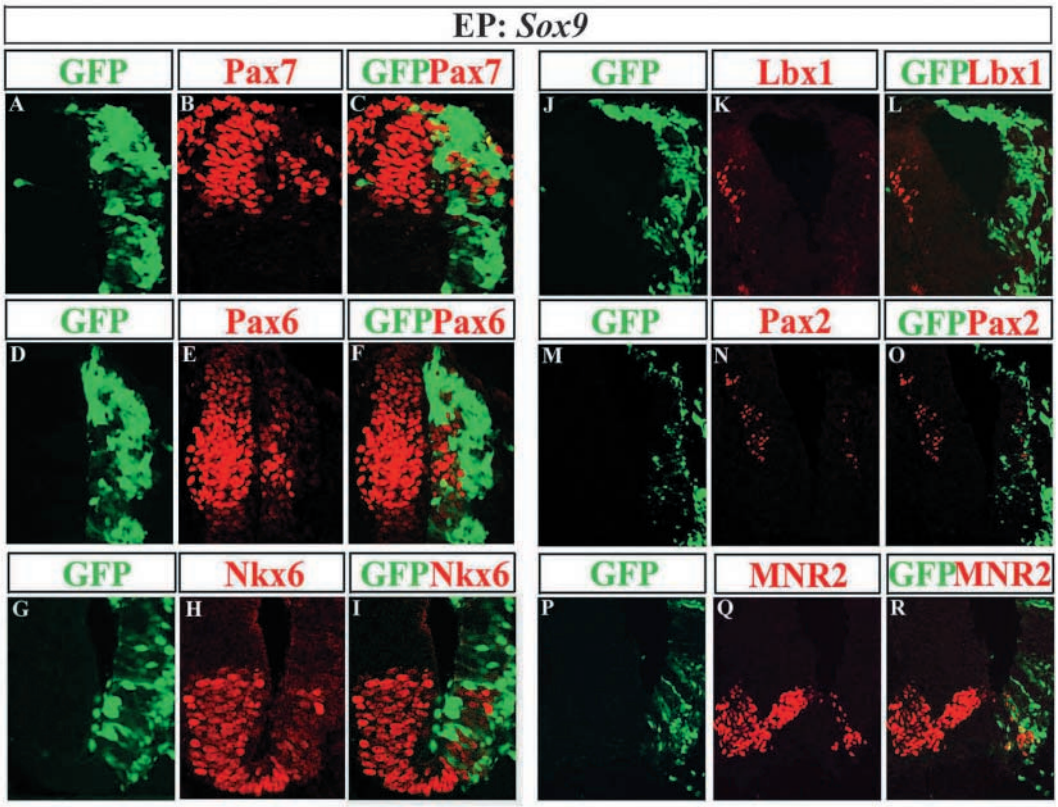
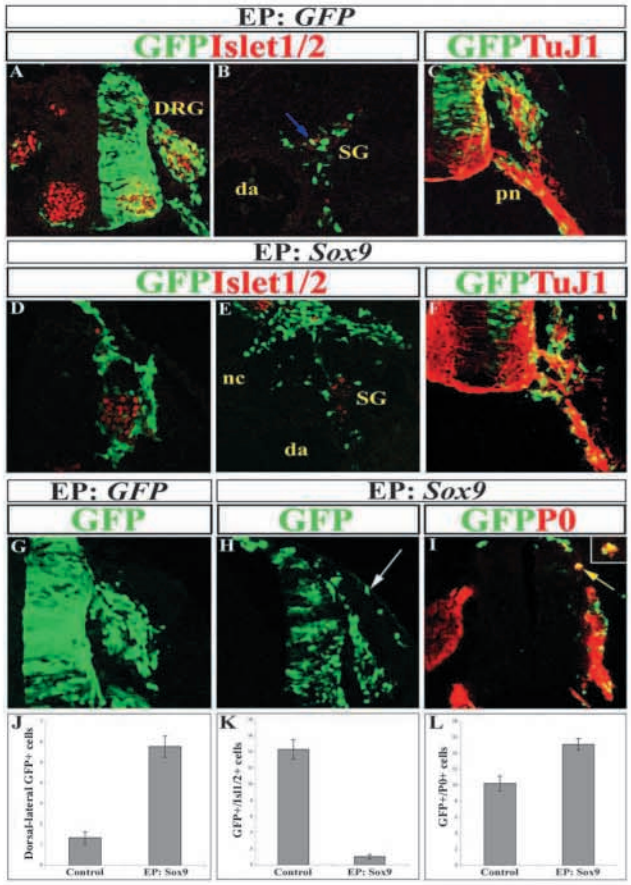


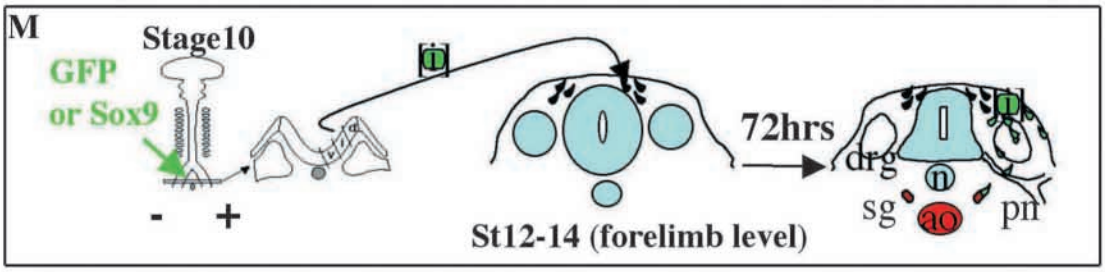
Fig. 5. Ectopic *Sox9* expression suppresses neurogenesis. Immunohistochemical detection of neural progenitor markers (A-I) and interneuron markers (J-R) on transverse sections of neural tubes 24 hours (A-I) and 48 hours (J-R) after electroporation with *Sox9*. Ectopic Sox9 suppresses *Pax7* (A-C), *Pax6* (D-F) and *Nkx6* (G-I) expression in the neural progenitor cells in a cell-autonomous manner. Moreover, neuronal differentiation is inhibited by *Sox9* expression. Induction of *Lbx1*-expressing interneurons in the dorsal neural tube is inhibited by *Sox9* expression (J-L), as are the induction of *Pax2*-expressing interneurons (M-O) and *HB9*/*MNR2*-expressing motor neurons (P-R). The effect of *Sox9* is cell autonomous, with untransfected cells differentiating into neuronal subtypes in a position-appropriate manner.

with GFP only, transfected cells could be observed in the dorsal root ganglion (Fig. 6A) and sympathetic ganglion (Fig. 6B), and along the peripheral nerve (Fig. 6C). Within ganglions, cells co-expressing GFP were found in both glial cells and neurons. Consistent with this, a significant proportion of GFP^+ cells expressed the neuronal markers *Isl1/2* (Fig. 6A,B,K, *n*=9) and *TuJ1* (Fig. 6C, *n*=9). In embryos electroporated with *Sox9*, transfected cells were also found along the peripheral nerve (Fig. 6F) and in the dorsal root (Fig. 6D) and sympathetic ganglions (Fig. 6E), but the distribution of cells differed markedly. $Sox9^+$ cells tended to be excluded from the core of the ganglions (Fig. 6D) and co-expression of neuronal markers and *Sox9*-expressing cells was not detected (Fig. 6D,E,K, *n*=9). Consistent with this, *Sox9*-transfected cells that expressed the Schwann cell marker P0 were detected (Fig. 6I,L; 8/9 embryos) (Bhattacharyya et al., 1991). In addition by 24 hpt, many *Sox9*-expressing cells were observed in the dorsal-lateral migration pathway characteristic of melanocyte differentiation (Fig. 6H,J; 9/9 embryos). At this stage in control embryos, few if any neural crest cells have entered this migratory pathway (Fig. 6G,J). These data suggest that a proportion of *Sox9*-expressing cells precociously enter the melanocyte migration stream. We therefore conclude that expression of *Sox9* in neural crest cells promotes melanocyte development, is permissive for glial cell development but is incompatible with neuronal differentiation.

Sox9-induced neural crest cells generate glial cells in vivo

The electroporation protocol used in these studies results in the transfection of endogenous premigratory neural crest, as evidenced by the distribution of GFP expressing cells in control embryos. In *Sox9*-transfected embryos, it is therefore not possible to distinguish between neural crest originating from the endogenous neural crest region and cells derived from ectopically generated neural crest. To examine whether ectopic neural crest induced by *Sox9* could generate differentiated neural crest derivatives, an electroporation and transplantation approach was taken. Stage 10 chick embryos were electroporated in ovo with *Sox9* or control vector and intermediate [i] neural explants from electroporated embryos were isolated 1-2 hours later and transplanted between the neural tube and somite of forelimb level stage 12-14 embryos. These embryos were then incubated for 72 hours and analysed (Fig. 6M). Using this approach, all GFP-expressing cells are derived from an electroporated [i] region and hence would not normally be expected to generate neural crest. Consistent with this, in control transplants of [i] regions expressing only GFP, transfected cells were observed in the position of the transplant (Fig. 6N-Q; 7/8 embryos) and many of these cells expressed *Pax7* (Fig. 6N; 6/7 embryos) and *Isl1/2* (Fig. 6Q; 5/7 embryos) markers of dorsal neural tube progenitors and neurons, respectively, and expression of the neural crest markers *HNK-1* (Fig. 6O; 7/7 embryos) or *P0* (Fig. 6P; 6/7 embryos) were not observed in transplanted cells. By contrast, in embryos that received transplants of cells transfected with *Sox9*, GFP^+ cells migrated away from the site of the transplant taking routes associated with migrating neural crest cells (Fig. 6R-U; 9/9 embryos). $Sox9^+$ cells were found within dorsal root ganglions (Fig. 6S-U) and sympathetic ganglions (Fig. 6R), along the ventral nerve (Fig. 6S,T) and in dorsal lateral positions underneath the ectoderm (Fig. 6S-U). Moreover, expression of *HNK-1* (Fig. 6S; 9/9 embryos) and *P0* (Fig. 6T; 9/9 embryos)





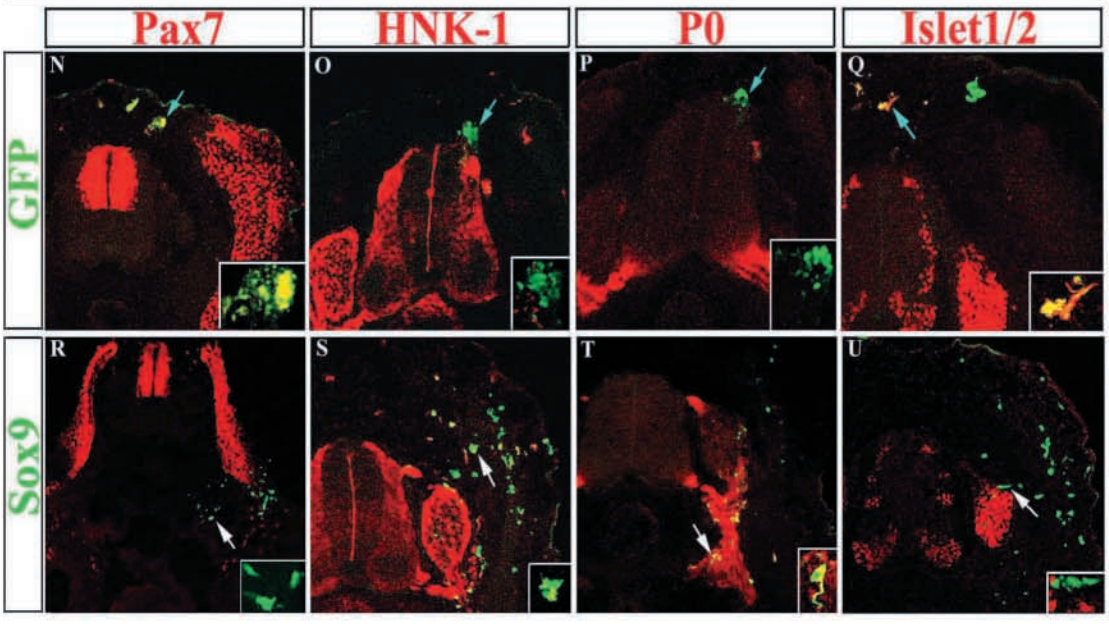


Fig. 6. Forced expression of Sox9 influences the fate of neural crest cells in the periphery. (A-F) Transverse sections of the neural tube electroporated with GFP alone (A-C) and *Sox9* IRES GFP (D-F) analysed 48 hpt. (A,B) GFP$^+$ neural crest cells migrate into core and peripheral regions of dorsal root ganglion (DRG) and sympathetic ganglion (SG), and some cells express the neuronal marker Islet1/2 (blue arrow). (C) GFP$^+$ cells are also observed along the peripheral nerve (pn) identified by β-tubulin (TuJ1) expression. (D,E) In a *Sox9*-electroporated embryo, GFP$^+$ cells are not observed coexpressing Isl1/2 in either the DRG (D) or SG (E) and cells are predominantly located in the periphery of the ganglia (D,E). (F) *Sox9*-transfected cells are also observed along the *TuJ1*$^+$ peripheral nerve. (G,H) Embryos electroporated with GFP alone (G) or *Sox9* (H) analysed 24 hpt. Cells transfected with only GFP migrate exclusively along the medial-lateral migration route leading to the DRG (G). By contrast, cells expressing *Sox9* migrate along both medial-lateral and dorsal-lateral migration routes (H) (white arrow). (I) Co-expression of *Sox9* and the gene encoding the Schwann cell marker protein zero (P0) is observed in neural crest cells (yellow arrow, inset) after 72 hpt. (J-L) Quantitative analysis of the effects of *Sox9* expression on neural crest cells fate (n=9, ±s.e.m.). Following electroporation of *Sox9*-IRES-GFP, the number of cells in dorsal-lateral migratory route increased compared with the GFP control (J), whereas the number of cells co-producing Islet1/2 was reduced (K), Moreover a small increase in the number of cells co-producing P0 was evident in *Sox9*-IRES-GFP-transfected embryos (L). (M) The experimental approach. Following electroporation of GFP alone or *Sox9*-IRES-GFP, neural plate explants [i] are dissected and transplanted into the region between the neural tube and somite at stage 12-14 of the chick embryos at forelimb level and incubated for 72 hours before processing. (N-Q) Cells from [i] regions transfected with GFP alone do not migrate away from the position of transplantation. Many of these cells express *Pax7* (N) and *Islet1/2* (Q) but few if any GFP$^+$ cells were observed expressing *HNK-1* (O) or *P0* (P). Blue arrows in (N-Q) indicate the high magnification of GFP$^+$ cells shown in the insets. (R-U) By contrast, *Sox9*-expressing cells show extensive migration from sites of transplantation. These cells do not express *Pax7* (R) or *Islet1/2* (U) but many express *HNK-1* (S) and *P0* (T). White arrows in (R-U) indicate the high magnification of the Sox9$^+$ cells shown in the insets. nc, notochord; da, dorsal aorta.

could be detected in *Sox9*-transfected cells and *Pax7* expression had been extinguished (Fig. 6R; 8/9 embryos). Consistent with the incompatibility of *Sox9* expression and neuronal differentiation, *Sox9*-transfected cells rarely if ever expressed neuronal markers (Fig. 6U and data not shown; 7/9 embryos). These data indicate that ectopic neural crest induced by Sox9 can differentiate into neural crest derivatives, notably glial cells.

Discussion

We provide evidence that the SoxE gene *Sox9* plays a central role in the development of the neural crest. Prospective neural crest cells express *Sox9* early in their development, and the forced expression of *Sox9* induces neural crest properties in neural progenitors. *Sox9* alone, however, does not efficiently induce the delamination of ectopic crest cells from the neural tube, consistent with the idea that this event is independent of the initial induction of neural crest. In migrating neural crest cells, *Sox9*-expressing cells exhibit characteristics of glial cells and melanocytes but are excluded from neuronal derivatives of neural crest. Altogether, our study indicates SoxE genes play two crucial roles in neural crest development: (1) in forming neural tissue, SoxE genes commit progenitors to the neural crest lineage; (2) subsequently, SoxE genes influence the differentiation of migrating neural crest cells.

Sox9 and induction of neural crest development

Forced expression of *Sox9* in the neural tube initiates a programme of neural crest development. Consistent with these data, loss of function analyses in *Xenopus* suggest that Sox9 is required for neural crest development (Spokony et al., 2002). Whether Sox9 is necessary for the specification of neural crest in other species remains to be determined. The overlapping production of the related proteins Sox8 and Sox10, and the demonstration that each SoxE gene is sufficient to induce neural crest differentiation suggests functional redundancy between SoxE genes that might partially or fully compensate for the loss of an individual family member. Thus, it is possible that only limited neural crest defects will be seen in *Sox9* loss-of-function mutants.

Sox9 is expressed in many cell types in addition to the neural crest (Zhao et al., 1997) and appears to play a role in the development of many tissues (da Silva et al., 1996; Bell et al., 1997). In each tissue, Sox9 is proposed to carry out a distinct biological function and to regulate a different subset of genes. Moreover, although *Sox9* expression is restricted to prospective neural crest regions of the early neural tube, it is subsequently expressed more broadly in neural progenitors, in which it appears to have a role in CNS glial development (Claus Stolt et al., 2003). It seems likely that this reflects changing competence of neural progenitors over time – early neural progenitors respond to Sox9 by neural crest induction, whereas later progenitors have lost their ability to do this. The transcriptional regulation by Sox genes usually requires DNA-binding cofactors, hypothesized to provide target specificity, that differ between tissues (Kamachi et al., 2000). In the case of Sox9, different cofactors have been identified in chondrocytes (Lefebvre et al., 1998) and genital ridge cells (de Santa Barbara et al., 1998). It is therefore possible that a partner necessary for neural crest induction is expressed in neural progenitors; the identity of this putative cofactor remains to be determined, but our data suggest that it is expressed throughout early neural progenitors but is subsequently downregulated so that later expression of *Sox9* no longer promotes neural crest induction.

Pathway of neural crest induction by Sox9

Neural crest induction has been divided into several sequential steps. Initially, prospective neural crest cells are segregated from dorsal neural progenitors by an inductive signal. Subsequently, these cells delaminate from the neural tube and begin their migration into the periphery (Le Douarin and Kalcheim, 1999). Our data indicate a role for SoxE genes at two distinct steps in neural crest development. In the first step, Sox9 acts cell autonomously downstream of the initial inductive event to induce a range of neural crest properties including the expression of *HNK-1*, *Slug*, *Cad6B*, *Cad7*, *FoxD3* and *Sox10*.

The temporal sequence of gene induction in *Sox9*-transfected cells is largely consistent with the temporal order of gene expression in endogenous neural crest. Sox9 rapidly and transiently induces factors characteristic of premigratory neural crest such as *Slug* and *Cad6B* (Nieto et al., 1994; Nakagawa and Takeichi, 1998). Markers of migratory neural crest, such as *Sox10*, *HNK-1* and *Cad7* (Cheng et al., 2000; Bronner-Fraser, 1986; Nakagawa and Takeichi, 1998) are induced more slowly and maintained at 24 hpt. These data are consistent with the idea that *Sox9* expression is an early response to neural crest induction signal and initiates the neural crest differentiation programme. One exception is *FoxD3*, the endogenous expression of which occurs in premigratory neural crest and migratory neural crest (Dottori et al., 2001; Kos et al., 2001). However, ectopic Sox9 does not induce *FoxD3* until 24 hpt, the time at which migratory neural crest markers are induced. This raises the possibility that *FoxD3* expression in neural crest cells can be divided into two phases – an early Sox9-independent phase and a later phase in which the neural crest differentiation programme initiated by Sox9 induces *FoxD3* expression. Given the evidence that forced expression of *FoxD3* is also sufficient to induce some aspects of neural crest differentiation (Dottori et al., 2001; Kos et al., 2001), it will be interesting to determine the epistatic relationship between *Sox9* and *FoxD3*.

In embryos transfected with *Sox9*, endogenous premigratory neural crest markers are downregulated 12 hpt earlier than would normally be expected. There is also a marked increase in the number of migratory neural crest cells originating from this region. These data are consistent with a model of neural crest development that involves the continuous recruitment of premigratory neural crest cells from adjacent neuroepithelium (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Selleck and Bronner-Fraser, 1995). In *Sox9*-transfected embryos, the transcriptional programme of premigratory neural crest is initiated in all *Sox9*-expressing cells simultaneously, accordingly all cells in the dorsal region of the neural tube commence neural crest differentiation synchronously. Thus, these cells pass through the premigratory stage by 12 hpt with the consequence that the pool of cells that could be progressively recruited to replenish the premigratory neural crest population is exhausted.

Coupling neural crest induction and delamination

In *Sox9*-transfected embryos, the depletion of cells from the

neural tube and the marked increase in the number of delaminating neural crest cells are only evident in dorsal regions. In intermediate and ventral neural tube regions, only a small proportion of *Sox9*-expressing cells delaminate. Consistent with this, expression of *HNK-1* and *Cad7* [markers normally restricted to migratory neural crest cells (Bronner-Fraser, 1986; Nakagawa and Takeichi, 1988)] is found within the neural tube of transfected embryos in cells with a pseudostratified epithelial organization. These data suggest that cells in ventral and intermediate regions of the neural tube are not competent to delaminate efficiently in response to neural crest induction. It is possible that emigration is restricted to the dorsal region by cell-intrinsic or -extrinsic signals constraining delamination in intermediate or ventral regions. Alternatively, it is possible that a signal is required dorsally to induce delamination directly. In support of this second idea, Sela-Donenfeld and Kalcheim have provided evidence that roof-plate-resident BMPs are necessary to promote the emigration of premigratory neural crest cells (Sela-Donenfield and Kalcheim, 1999). Thus, the dorsal restriction of delamination of *Sox9*-induced neural crest might reflect the range of effective BMP signalling that is sufficient to promote delamination.

RhoB has been identified as a cell intrinsic determinant of neural crest delamination (Liu and Jessell, 1998). Ectopic expression of *RhoB* is not observed in *Sox9*-transfected embryos raising the possibility that the lack of RhoB accounts for the low frequency of delamination in intermediate and ventral regions of the neural tube. Moreover, the dorsal region (in which forced expression of *Sox9* does result in robust increased neural crest migration) encompasses the region of endogenous *RhoB* expression. The upregulation of *RhoB* therefore appears to demarcate a region of cells competent to delaminate and the expression of *RhoB* or factors with a similar expression profile might provide the molecular mechanism that triggers neural crest delamination. Thus, the coordinated induction of *Sox9* and *RhoB* in dorsal regions of the neural tube might act to couple the sequential steps of neural crest induction and delamination during neural crest development.

Although ectopic delamination of *Sox9*-expressing cells in the intermediate and ventral neural tube is inefficient, it can still be observed, albeit at low frequency. Our findings suggest that Sox9 might induce delamination in a RhoB-independent manner. However, we cannot rule out the possibilities that RhoB induction occurs at a low level in our experiments or that other members of the Rho family partially substitute for the lack of RhoB (Liu and Jessell, 1998). Dottori et al. (Dottori et al., 2001) have suggested that ectopic expression of *FoxD3* induces neural crest delamination in a RhoB-independent manner but, in these experiments, similar considerations also need to be taken into account; emigration of neural crest was relatively inefficient and *HNK-1* expression was prominent in the neural tube.

SoxE-expressing neural crest acquires properties of glial cells and melanocytes but not neurons

Trunk neural crest cells adopt one of a range of potential fates and these can be distinguished by the migration pathway, morphology and gene expression profile of the cell (Le Douarin and Kalcheim, 1999). Our data suggest that *Sox9* is expressed by the progenitors of all neural crest derivatives and *Sox9*-expressing cells migrate along the routes of normal neural crest migration, and are subsequently to be found residing in sympathetic and dorsal root ganglions, peripheral nerves and underneath the ectoderm. The expression of a SoxE gene in these migrating neural crest cells, however, biases the differentiation pathways taken by these cells. *Sox9*-positive cells are excluded from neuronal cell types, suggesting that the expression of *Sox9* is incompatible with neuronal differentiation. *Sox9*-expressing cells do, however, acquire the characteristics of glial cells, as shown by the presence of *Sox9*-expressing cells along the peripheral nerve and the identification of *Sox9*$^{+}$ cells that co-express *P0*. *Sox9*-expressing cells were also observed entering the dorsal lateral migration pathway, underlying the ectoderm. This pathway is characteristic of melanocytes (Reedy et al., 1998). In the chick, neural crest cells typically migrate along this route only at later stages of development, and these cells are restricted to generating melanocytes (Reedy et al., 1998). The finding of increased numbers of *Sox9*-expressing cells migrating via this pathway in transfected embryos raises the possibility that *Sox9* induces the precocious development of melanocytes.

The restriction of *Sox9*-expressing cells to a subset of neural crest derivatives might indicate that Sox9 induces crest progenitors with restricted differentiation potential. However, most of the lineage analysis (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989) and in vitro culture studies (Stemple and Anderson, 1992) suggest that the fate of neural crest cells is not restricted prior to their emergence from the neural tube. The Wnt signalling pathway has been shown to influence the differentiation of melanocytes (Ikeya et al., 1997; Hari et al., 2002). The induction of *Wnt3a* by Sox9 might therefore direct a proportion of *Sox9*-expressing neural crest cells to this lineage. Alternatively, this activity of *Sox9* might reflect a later role for SoxE group genes in the periphery. *Sox10* has been implicated in regulating glial and pigment cell differentiation (Britsch et al., 2001; Dutton et al., 2001). We demonstrate that *Sox9* induces *Sox10* expression in ectopic neural crest 12-24 hpt, so it is possible that upregulation of *Sox10* results in the induction of particular fates in the periphery at the time cells differentiate. Alternatively, functional equivalency between SoxE genes might account for the ability of *Sox9* to direct neural crest cells towards non-neuronal fates. In this view, *Sox9* takes over the role of *Sox10* and directly controls the fate decisions. In conclusion, our study indicates that *Sox9* plays an important role in the developmental programme of neural crest cells, initially inducing neural crest differentiation and then biasing the differentiation of migrating neural crest cells to non-neuronal cells types.

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