LSox5 regulates RhoB expression in the neural tube and promotes generation of the neural crest

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Summary

Members of the Sox family of transcription factors are involved in a number of crucial developmental processes, including sex determination, neurogenesis and skeletal development. LSox5 is a member of the group D Sox factors that, in conjunction with Sox6 and Sox9, promotes chondrogenesis by activating the expression of cartilage-specific extracellular matrix molecules. We have cloned the chicken homologue of *LSox5* and found that it is initially expressed in the premigratory and migratory neural crest after *Slug* and *FoxD3*. Subsequently, the expression of *LSox5* is maintained in cephalic crest derivatives, and it appears to be required for the development of the glial lineage, the Schwann cells and satellite glia in cranial ganglia.

Misexpression of *LSox5* in the cephalic neural tube activated *RhoB* expression throughout the dorsoventral axis. Furthermore, the prolonged forced expression of *LSox5* enlarged the dorsal territory in which the neural crest is generated, extended the 'temporal window' of neural crest segregation, and led to an overproduction of neural crest cells in cephalic regions. In addition to HNK-1, the additional neural crest cells expressed putative upstream markers (*Slug*, *FoxD3*) indicating that a regulatory feedback mechanism may operate during neural crest generation. Thus, our data show that in addition to the *SoxE* genes (*Sox9* and *Sox10*) a *SoxD* gene (*Sox5*) also participates in neural crest development and that a cooperative interaction may operate during neural crest generation, as seen during the formation of cartilage.

Supplemental data available online

Key words: LSox5, Neural crest, RhoB, Peripheral glia, Chick

Introduction

Since the discovery of Sry (Gubbay et al., 1990), many transcription factors have been characterised and included in the Sox family (Sry-related HMG box), based on their homology to the HMG DNA-binding domain. These proteins are involved in a number of developmental processes, making the Sox family one of the most prolific of its kind with more than 30 vertebrate members recognised to date. Through the phylogenetic analysis of the HMG box, Sox family members have been classified into 11 groups (Bowles et al., 2000). Together with Sox6 and Sox13, Sox5 belongs to group D.

SoxD genes contain more exons than do other Sox genes (Schepers et al., 2002). Additional complexity is introduced through tightly regulated mRNA processing, the functional significance of alternative splicing being highlighted by the differential expression of the distinct isoforms. Indeed, a short variant of Sox5 (SSox5) is found only in adult testis, while longer isoforms (LSox5) are expressed in other tissues during mammalian development (Denny et al., 1992; Lefebvre et al., 1998; Hiraoka et al., 1998). LSox5 variants share common structural features with other group D members, including a leucine zipper and glutamine-rich regions thought to be involved in dimerisation (Hiraoka et al., 1998; Lefebvre et al., 1998). In the mouse, LSox5/Sox6 heterodimers cooperate with *Sox9* to activate the *Col2a1* gene, which encodes type II collagen, an extracellular matrix component that is essential for chondrogenesis (Lefebvre et al., 1998). Indeed, it is in this process that LSox5 has been most thoroughly studied. Mutations in *SOX9* produce campomelic dysplasia, a syndrome that is often associated with autosomal XY sex reversal and involves the severe malformation of most cartilage-derived structures (Meyer et al., 1997). Furthermore, mouse chimeras containing *Sox9*$^{-/-}$ embryonic stem cells inactivate early cartilage markers, including the *Col2a1* gene (Bi et al., 1999), a phenomenon that is also observed in *Sox5*, *Sox6* double mutant embryos (Smits et al., 2001). This scheme is further clarified by the severe downregulation of *LSox5* and *Sox6* expression produced after *Sox9* inactivation using the Cre/LoxP system in chondrogenic cell lineages, thus demonstrating that *LSox5* and *Sox6* are genetically downstream of *Sox9* in chondrocytes (Akiyama et al., 2002). Interestingly, in the chick, Sox9 has been implicated in the differentiation of the neural crest (Cheung and Briscoe, 2002) and here we characterise chick LSox5 and show its participation in cranial neural crest development.

The neural crest is a cell population that originates at the boundary between the neural plate and the prospective epidermis. Once specified, the cells of the neural crest delaminate from the neural folds/neural tube by undergoing a process of epithelium to mesenchyme transition (EMT). These cells then migrate along characteristic pathways and differentiate into a wide variety of derivatives upon reaching

their destination, including neurons and glia of the peripheral nervous system, pigment cells, craniofacial cartilage and bone (LeDouarin and Kalcheim, 1999). Diffusible factors of the BMP, BMP antagonists, FGF and WNT families, together with retinoic acid, seem to direct the first steps of neural crest induction (Liem et al., 1995; Selleck et al., 1998; Garcia-Castro et al., 2002; Villanueva et al., 2002), although different transcription factors are thought to interpret these extracellular signals. Indeed, the winged-helix family member FoxD3 and Sox9 have been implicated in the induction of the neural crest (Kos et al., 2001; Dottori et al., 2001; Cheung and Briscoe, 2003); and members of the Snail family of transcription factors, Snail and Slug, are required to trigger the EMT (reviewed in Nieto, 2002). The neural crest derivatives become committed at different stages of their development and some transcription factors seem to direct this lineage commitment. At low concentrations, Sox10 apparently maintains the multipotency of the crest cells and at higher doses it inhibits neuronal differentiation, favouring the generation of peripheral glia and melanoblasts (Kim et al., 2003; Paratore et al., 2001). by contrast, FoxD3 represses melanogenesis (Kos et al., 2001; Dottori et al., 2001) and it has been demonstrated that Sox9 is necessary for the determination of the chondrogenic lineage in cranial neural crest cells (Mori-Akiyama et al., 2003).

We have characterised chick LSox5, which shows a high degree of similarity to its mammalian counterpart and an identical HMG box. Two splice variants were found that contain specific structural motifs susceptible to post-translational modification. We show that LSox5 is expressed in premigratory cranial neural crest cells and that following delamination, LSox5 expression coincides with a characteristic pattern of crest migration. High expression levels are maintained in the crest-derived components of cranial glia, including Schwann cells and satellite glia. Misexpression of LSox5 in the midbrain and the hindbrain provoked the rapid, cell autonomous upregulation of *RhoB*. This misexpression of LSox5 also led to an extension of the dorsal territory and of the developmental window in which the neural crest is produced, and augmented the generation of cephalic neural crest. The less immediate effects of LSox5 include the upregulation of other neural crest markers, such as *Slug*, *FoxD3* and *Sox10*. Thus, we propose that LSox5 participates in the generation of the cranial neural crest and in the subsequent differentiation of the cranial glia lineage.

Materials and methods

Embryos

Fertilised chicken eggs were purchased from Granja Santa Isabel (Córdoba, Spain). Eggs were incubated, opened and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Cloning and sequencing of chicken *LSox5*

Chicken *LSox5* was isolated in a subtractive screen to isolate genes involved in the early stages of brain development. A cDNA subtraction library was generated with the 'Clontech PCR select-cDNA subtraction kit' (Clontech, catalog number K1804-1), using the anterior region of stage 8 and stage 11 chick embryos as the 'driver' and 'tester' populations, respectively. One of the clones isolated in the screen was a partial cDNA for *LSox5*, and the full-length cDNAs of *LSox5-I* and *LSox5-II* (2.3 and 2.4 kb, respectively) were obtained by PCR walking using a stage14 lambda zap cDNA library. The nucleotide sequence of *LSox5* appears in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the Accession Numbers AJ626988 and AJ626989. A putative short isoform (*SSox5*) results from the deletion of the first 1248 coding nucleotides of *LSox5-II*. Hence, we analysed the expression of the alternatively spliced *LSox5* isoforms by RT-PCR using the flanking oligonucleotides: sense 5′-CTCCCAGCCTTTCACCTTCCC-3′; and antisense 5′-GCTTTCT-GGAGTCCCTTTTAT-3′.

In ovo electroporation

Full-length *LSox5-II* (772 amino acids) and *SSox5* (356 amino acids) cDNAs were cloned into a pCX vector (Niwa et al., 1991) to be used in electroporation assays. Each plasmid (4 mg/ml) was injected between the neural folds of stage 8 chicken embryos together with pCX-*EGFP* (0.5 mg/ml), and the embryos were immediately electroporated as described previously (Itasaki et al., 1999) with some modifications. Electroporation was performed with a TSS20 Intracel square pulse generator, programmed to deliver five 50 mseconds, 15 V pulses at 4 Hz, through custom-made platinum electrodes. The eggs were sealed and allowed to develop for another 6-7, 12 or 20-24 hours, at which point the embryos were removed and processed for in situ hybridisation or immunohistochemistry. Control electroporation assays were performed by injecting pCX-*EGFP* (4.5 mg/ml) alone.

Whole mount in-situ hybridisation

Single or double whole-mount in situ hybridisation was carried out as described (Nieto et al., 1996). For double hybridisation, one probe was labelled with digoxigenin-UTP and the second with fluorescein-UTP. The probes were detected with anti-digoxigenin or anti-fluorescein antibodies coupled to alkaline phosphatase, and visualised with NBT-BCIP (digoxigenin) or INT-BCIP (fluorescein; all reagents and antibodies supplied by Roche). The *LSox5* probes were prepared from the full-length *LSox5-II* cDNA isoform. The fluorescein-labelled *LSox5* probe was often used to control for electroporation without masking the blue staining of the probe under analysis. Embryos were then photographed prior to embedding in gelatin or agarose for vibratome sectioning.

Anti-LSox5 generation and immunohistochemistry

The *LSox5-II* cDNA was cloned into the pRSET plasmid (Invitrogen), from which a fusion protein was generated to immunise rabbits and obtain antisera. Protein extracts from embryonic fibroblasts stably infected with RCAS vectors expressing *LSox5-I* or *LSox5-II* were analysed by western blotting to test the affinity and specificity of the sera. One serum, 32A-III, specifically recognised LSox5 in western blots and produced a pattern of immunostaining comparable with that obtained by in situ hybridisation (not shown). This serum was used throughout this work. For immunohystochemistry, 10 μm cryostat or 40 μm vibratome agarose sections were permeabilised with 0.5% Triton X-100 (USB), blocked with 10% FBS and incubated overnight at 4°C with the primary antibody. After washing, the cryostat sections were incubated for 1 hour with secondary antibodies or overnight in the case of agarose vibratome sections. The primary antibodies were used at the following concentrations: LSox5, 1:4000; EGFP, 1:1000 (Molecular Probes); Laminin 1, 1:1000 (Sigma); Pax7, 1:1000 (DSHB) (Ericson et al., 1996); Slug, 1:1000 (62.1E6, DSHB) (Liem et al., 1995); P_0, 1:1000 (1E8, DSHB) (Bhattacharyya et al., 1991); HNK-1, 1:4000 (prepared from a cell line obtained from ATCC); Islet-1/2, 1:1000 (40.2D6, DSHB) (Ericson et al., 1992). Cy2- or Cy3-conjugated secondary antibodies were used (Jackson; 1:1000 dilution). HNK-1 whole-mount immunohistochemistry was performed as described (Nieto et al., 1996).

Results

Two isoforms of the transcription factor LSox5 expressed in chick embryos

In an attempt to isolate genes involved in the early stages of

chick brain development, we have identified clones corresponding to chick *LSox5* in a subtractive screen where cDNAs from stage 8 chick embryos were compared with those isolated from stage 11. cDNA clones that showed a strong similarity to mammalian *LSox5* and that were more intensely expressed at stage 11 were identified. Then, a full-length cDNA was generated by PCR cDNA walking, which rendered two distinct isoforms. The longer *LSox5-II* differed from the shorter *LSox5-I* sequence because of a 105 bp insertion that encoded additional amino acids at position 56 (the amino acid sequence of chicken LSox5 and its alignment with the human orthologue is shown in Fig. S1 at http://dev.biologists.org/cgi/content/full/131/18/4455/DC1). By comparing the *LSox5* cDNA sequence with public database entries, we mapped the chicken *Sox5* gene to chromosome 1 and established an exon/intron organisation very similar to that described for its mammalian orthologues (Ikeda et al., 2002) (the location and sequence of exon/intron boundaries is shown in Table S1 at http://dev.biologists.org/cgi/content/full/131/18/4455/DC1).

The two cDNA isoforms isolated in the chick encode proteins of 737 and 772 amino acids, predicted to be of 80.99 and 84.95 kDa, respectively. The amino acid sequences are highly similar to their mammalian counterparts, LSox5-II sharing 95.7% and 93% of its residues with its human and mouse orthologues, respectively. The HMG box is identical in all three species, but out of this region, the chick LSox5-II contains 113 serine/threonine residues, 10% more than its mammalian orthologues. Strikingly, the majority of the additional S/T residues are concentrated between amino acids 426-456, and within this region, four serines and one threonine are concentrated in a short nine amino acid stretch. In silico screening of EST databases, together with the analysis of the corresponding genomic entries has shown that the inclusion of this serine-rich domain (SRD) varies across species. It is constitutively incorporated in zebrafish and fugu LSox5 polypeptides, absent in human, mouse and rat, and it may be alternatively spliced in the chicken (see Fig. S2 at http://dev.biologists.org/cgi/content/full/131/18/4455/DC1 shows the DNA sequence at the intron-exon boundary of different species, providing a molecular basis for this diversity). These phylogenetic variations may reflect the evolution of mechanisms that regulate LSox5 activity which involve differential phosphorylation.

We analysed the expression of the two distinct *LSox5* isoforms by RT-PCR on cDNA samples from stages 9-23, with a pair of primers flanking the inserted domain. Two bands corresponding in size to that predicted for each isoform were detected at all stages and at the same intensity. Furthermore, in western blots of whole embryo protein extracts, a serum generated against LSox5 also detected two bands of similar intensity and with apparent molecular masses slightly higher than those predicted from the primary structure (the expression of *LSox5* as obtained by both RT-PCR and western blot is shown in Fig. S1 at http://dev.biologists.org/cgi/content/full/131/18/4455/DC1). Thus, we detected LSox5 in embryos at all the stages examined and no differences in expression could be detected between the two isoforms during development, either by RT-PCR or immunoblotting. Nevertheless, as these studies were performed on whole embryos, we cannot exclude the possibility that local differences do indeed exist.

LSox5 is expressed in premigratory and migratory cephalic neural crest cells

The long splice variants derived from the *Sox5* gene were first identified in the mouse. They have been shown to play a crucial role in chondrogenesis (Lefebvre et al., 1998) and to be expressed in the developing pancreas, as well as in a variety of adult human tissues (Ikeda et al., 2002; Lioubinski et al., 2003). At early stages of chick development, *LSox5* mRNA appeared to be distributed in the neural crest and its derivatives (Fig. 1). The expression of *LSox5* was first detected at stage 7 in the neural folds (Fig. 1A), closely following the onset of the expression of other neural crest markers such as *Slug* (data not shown) and *FoxD3* (Kos et al., 2001). At all the stages analysed, *LSox5* was also expressed at high levels in the notochord (Fig. 1C).

Within the neural plate, the expression of *LSox5* was dependent on its anteroposterior location, as transcripts were found both dorsally and ventrally at prosencephalic levels (Fig. 1B), whereas at more caudal levels they were restricted to the most dorsal region where the neural crest forms (Fig. 1C). As the neural folds extended caudally, so did the dorsal restriction of *LSox5* expression. At stages 8 and 9, *LSox5* continued to be expressed in the anterior prosencephalon, although this region does not produce neural crest (Fig. 1D,F) and nor were neural crest markers such as *Slug* and *FoxD3* expressed here (Fig. 1E) (Kos et al., 2001). At later stages, *LSox5*-expressing cells became migratory (Fig. 1G) and were found among the population of mesencephalic and rhombencephalic migratory crest cells (Fig. 1I). *LSox5* expression then progressively diminished in the dorsal cephalic neural tube caudal to the forebrain, coincident with its apparition in cells that were migrating along well-defined neural crest pathways (Anderson and Meier, 1981; Tosney, 1982; Lumsden et al., 1991).

To confirm whether the migratory cells that express *LSox5* were neural crest cells, we examined the expression of Slug, a marker of the premigratory and the migratory neural crest population (Fig. 1J) (Nieto et al., 1994; del Barrio and Nieto, 2004). Within the neural tube, all cells that expressed LSox5 also expressed Slug (Fig. 1J-L), confirming that LSox5 is expressed in premigratory neural crest cells. Nevertheless, a subpopulation of cells only expressed Slug, indicating that LSox5 is not common to all premigratory crest cells. In the early stages of migration, many cells expressed both these proteins, although as migration proceeded, LSox5 expression augmented while the expression of Slug diminished (del Barrio and Nieto, 2004). The onset of *Slug* expression prior to that of *LSox5* indicated that Slug precedes LSox5 in the genetic cascade at play during neural crest development.

LSox5 expression is maintained in the glial lineage of the cephalic peripheral nervous system

We then compared the expression of LSox5 with that of other neural crest markers and its distribution in neural crest derivatives. RhoB is a small GTPase implicated in the delamination of the neural crest that lies downstream of Slug (Liu and Jessell, 1998; del Barrio and Nieto, 2002). *RhoB* mRNA is expressed in a small population of premigratory neural crest cells and in the early migratory cells. Its expression pattern is very similar to that of *LSox5* in the premigatory neural crest cells, suggesting that at a particular stage of

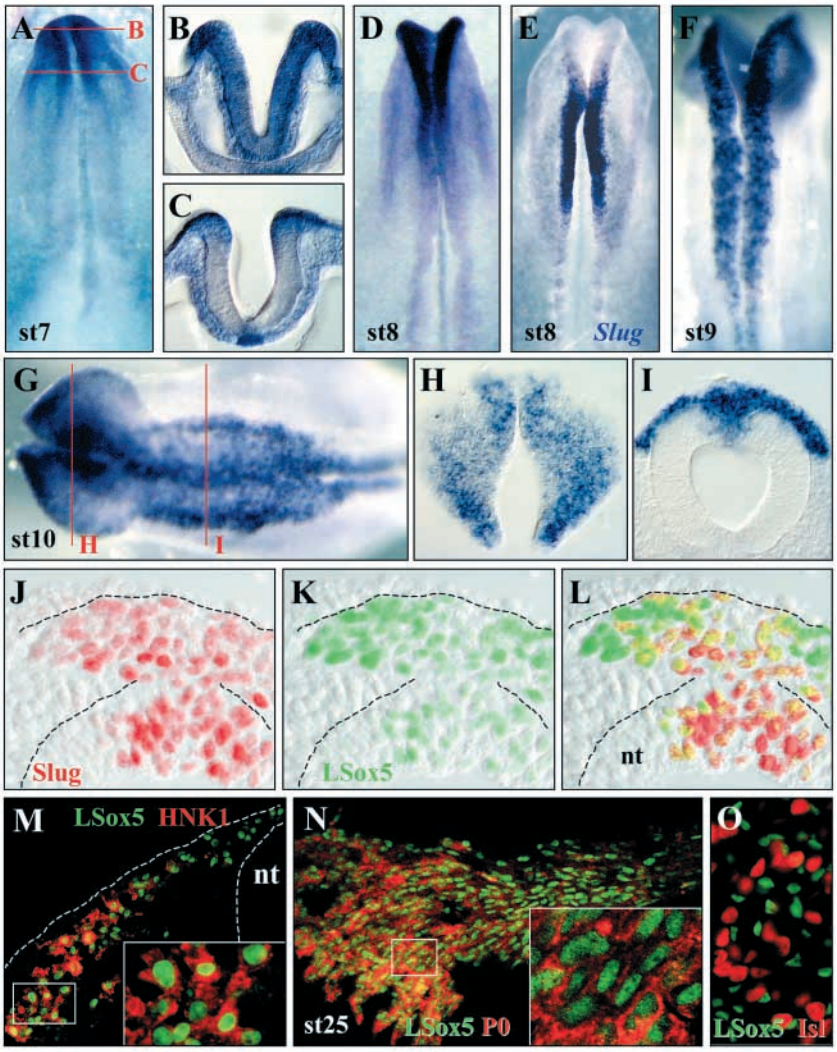

Fig. 1. Expression patterns of *LSox5* in the cephalic region of chick embryos. Dorsal views of stage 7 (A), 8 (D), 9 (F) and 10 (G) embryos show the expression of *LSox5* mRNA in the neural folds/neural tube, following a pattern compatible with premigratory and migratory cephalic neural crest. Transverse sections of stage7 (B,C) and stage 10 (H,I) embryos show *LSox5* expression all along the dorsoventral axis of the neural tube at prosencephalic levels (B,H), and a restriction to the most dorsal region at more caudal levels (C,I). (E) A dorsal view of a stage 8 embryo showing the expression of *Slug* in territories competent to form neural crest, and its absence from the non-crest producing prosencephalic regions. (J-L) A transverse section of a stage 10 embryo at the hindbrain level labelled with both anti-LSox5 (green) and anti-Slug (red) antibodies. Only a subpopulation of the cells within the neural tube (nt) express both Slug and LSox5, whereas all the migratory cells express both genes. LSox5 expression increases along the cell migratory tracts, while Slug expression diminishes in some migratory cells. (M) A transverse section of a stage 10 embryo at the level of the rostral hindbrain shows that as migration proceeds, LSox5 immunoreactive cells (green) acquire the HNK1 epitope (red). (N) Parasagittal section of a stage 25 embryo showing the proximal segment of the oculomotor nerve. LSox5 expression (green) is high in the precursors of the Schwann cells, where it coincides with the P_0 marker. (O) Section through the ophthalmic lobule of the trigeminal ganglion of an E5.5 (stage 28) embryo double stained for Islet (red) and LSox5 (green). The small nuclei, morphologically associated with satellite glia, express LSox5, while those of the neuroblasts express Islet.

development they may be co-expressed in neural crest cells. The glycoprotein epitope HNK1 (Tucker et al., 1984) is found in the majority of migratory neural crest cells (Le Dourain and Kalcheim, 1999; del Barrio and Nieto, 2004). Although most migratory neural crest cells were labelled for both HNK1 and LSox5 (Fig. 1M), an early migratory cell population contained LSox5 alone.

In the head, all the peripheral glia and most neurons in both the sensory and autonomic ganglia are lineages derived from the neural crest (reviewed by LeDourain and Kalcheim, 1999). The population of peripheral glia includes satellite glia of the cranial ganglia and the Schwann cells associated with the cranial nerves. Our immunohistochemical analysis revealed that LSox5 expression was maintained in both these glial cell types. In Schwann cell precursors that ensheath the cranial nerves, LSox5 is co-expressed with P_0, an early marker of both myelinating and nonmyelinating cells (Bhattacharyya et al., 1991) (Fig. 1N). In the cranial ganglia, anti-LSox5 labelled small rod-shaped nuclei whose morphology indicated that they might correspond to satellite glia. This population of LSox5-positive cells was clearly different from the neuroblasts that express Lim-domain factors of the Islet class (Fedtsova et al., 2003) (Fig. 1O).

LSox5 misexpression in the cephalic neural tube augments the production of neural crest

To investigate whether LSox5 plays a role in the migration and/or differentiation of the neural crest, we ectopically induced expression of the longest LSox5-II isoform on the right-hand side of the neural tube. This was achieved by electroporating pCX-*LSox5* (together with pCX-*EGFP* as a marker of transformation) into the cephalic region of stage 8 embryos. After 20-24 hours, uniform EGFP labelling was detected on one side of the neural tube and in the characteristic streams of migratory cranial neural crest (Fig. 2A). A similar distribution of ectopic LSox5 was observed by immunohistochemistry (Fig. 2B,C). Under these conditions, the expression of HNK1 increased on the electroporated side, particularly in the post-otic regions, although a similar effect was also observed at different cranial levels (Fig. 2D,E). Moreover, many of the additional HNK1-labelled cells were located at more retarded positions along the migratory pathway, and ectopic epithelial cells were also labelled by HNK1 throughout the electroporated side of the neuroepithelium (Fig. 2F-H). None of these effects was observed when pCX-*EGFP* alone was electroporated (not shown).

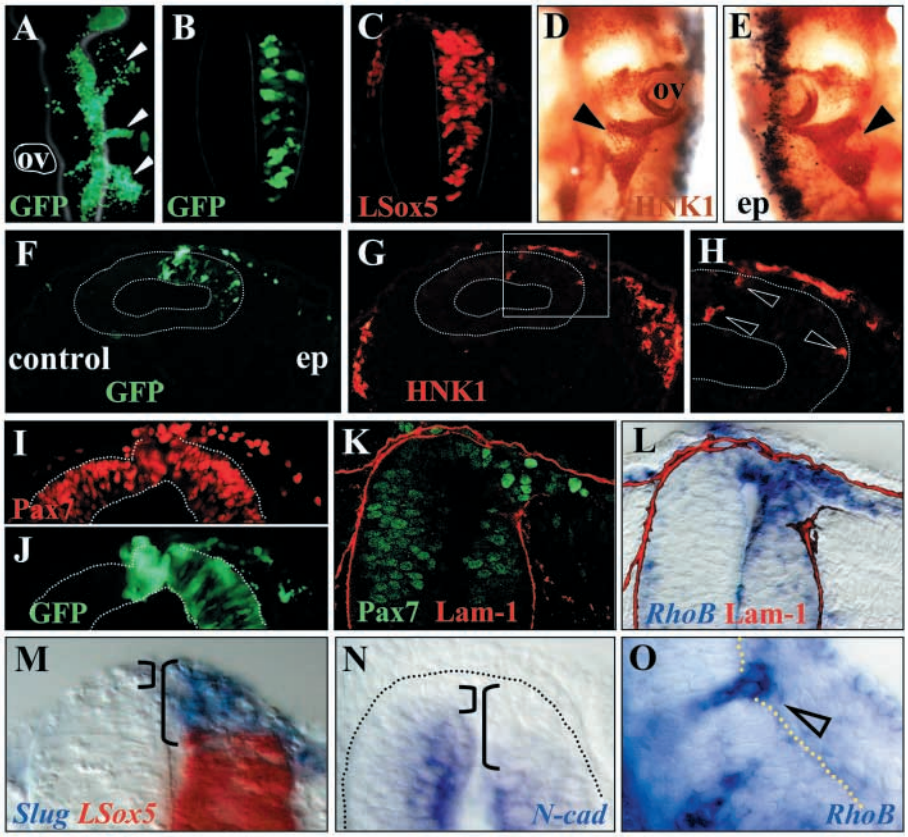

Fig. 2. *LSox5* misexpression in the cephalic neural tube augments the neural crest population. (A) Dorsal view of an embryo 20 hours after co-electroporation with pCX-*LSox5* and PCX-*EGFP* on the right-hand side of the cephalic neural tube. The EGFP expression is seen on the treated side and in the emerging streams of migratory neural crest cells (white arrowheads). (B,C) Transverse sections of an electroporated embryo showing the coincident expression of EGFP and ectopic LSox5 all along the dorsoventral axis of the right hand side of the neural tube. (D,E) Lateral views of an electroporated embryo immunostained for HNK1 where the increase in HNK1-positive migratory cells in the electroporated (ep) side can be observed, particularly in the circumpharyngeal stream (black arrowhead). (F,G) A transverse section at the level of the caudal hindbrain shows the supernumerary HNK1-positive migratory cells (red) in the electroporated side of the embryo. (H) A magnification of G shows the appearance of ectopic HNK1-positive cells within the treated side of the neural tube (arrowheads). (I,J) At the hindbrain level, in a double immunostained transverse section, the overexpression of Pax7 (red) in cells expressing EGFP (green) can be seen in both neuroepithelial and migratory cells on the *LSox5* transfected side. (K,L) An overlay of *RhoB* hybridisation and double immunostaining for Pax7 (green) and laminin 1 (red) shows that severe damage of the basement membrane coincides with the expanded region of neural crest delamination on the electroporated side of the neural tube. (M,N) Transverse sections of electroporated embryos hybridised for *LSox5* and *Slug* (M), and N-cadherin (N). The territory competent for neural crest generation extends ventrally on the electroporated side, as judged by the expanded expression of *Slug* (M) and the coincident inhibition of N-cadherin expression (compare the length of the brackets on both sides of the neural tube). (O) At more ventral regions of the neural tube, scattered cells could be detected that ectopically expressed *RhoB* on the treated side of the neural tube, breaking the basal lamina and invading into the lateral mesenchyme (open arrowhead). ov, otic vesicle.

The additional population of migratory cells were very probably bona fide neural crest cells, as they also expressed premigratory neural crest markers. Indeed, we found a dramatic increase in the number of Pax7-, *RhoB*- and *Slug*-expressing cells on the electroporated side of the dorsal neural tube (Fig. 2I-K, L and M, respectively). In addition to neuroepithelial cells in the dorsal neural tube, Pax7 is also expressed in a population of cranial neural crest migrating from the mesencephalon and rhombomeres 1, 3 and 5 (Kawakami et al., 1997). Coincidently, N-cadherin, the expression of which disappears in delaminating crest cells (Nakagawa and Takeichi, 1995), was also diminished in this region (Fig. 2N). Finally, the basement membrane was disrupted, indicating that these crest cells could undergo the epithelial-mesenchyme transition and delaminate (Fig. 2K,L). The misexpression of *LSox5* also generated some scattered *RhoB*-expressing cells at more ventral locations within the electroporated tube, and some of these cells appeared to delaminate from this ectopic site (Fig. 2O). However, ectopic expression of *Slug* was never detected at intermediate or ventral regions of the tube (Fig. 2M). In summary, ectopic overexpression of *LSox5* in the neural tube upregulated Pax7, *Slug* and *RhoB* expression dorsally, expanding the delaminating domain and increasing the migratory neural crest population. More ventrally, some ectopic *RhoB*-positive cells were generated that did not express *Slug*, although some of them seemed capable of exiting the neural tube.

The induction of *RhoB* is an immediate response to *LSox5* misexpression

Under normal conditions, the onset of *Slug* expression occurs before that of *LSox5*. Considering that the onset of *LSox5* and *RhoB* expression occurs within a similar timescale, and that *LSox5* is able to induce *RhoB* in areas where *Slug* was not induced (Fig. 2O), we examined whether *LSox5* could induce *RhoB* within short periods of time and whether this was independent of *Slug* induction. On the transformed side of the neural tube, we could identify a number of cells that expressed EGFP and LSox5 6-7 hours after electroporation (Fig. 3A,B). In these embryos, ectopic *RhoB* expression was also detected in the intermediate and ventral regions of the neural tube (Fig. 3C), but no abnormal *Slug* expression could be observed (not shown). Moreover, no ectopic cells expressing *RhoB* were observed in the contralateral side of the tube or in control embryos electroporated with pCX-*EGFP* alone (not shown). The rapid appearance of ectopic cells expressing both LSox5 and *RhoB* suggested that the forced expression of *LSox5* directly induced *RhoB* expression in the neural tube (Fig. 3D-

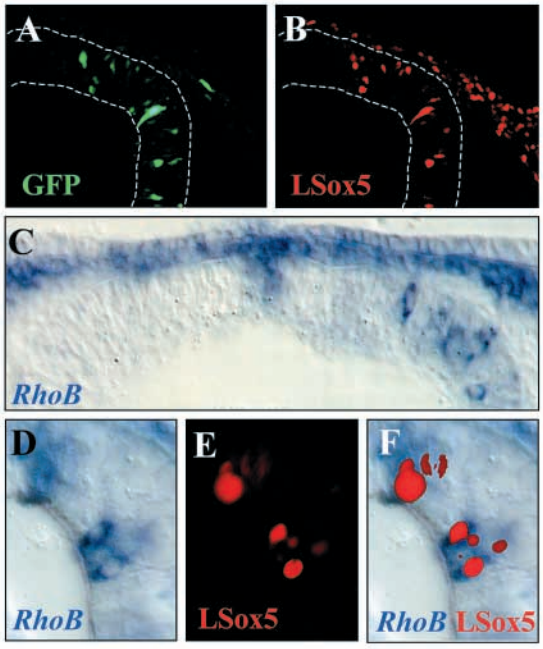

Fig. 3. Rapid activation of *RhoB* after ectopic LSox5 expression in the cephalic neural tube. Expression of EGFP (A); LSox5 (B,E,F) or *RhoB* (C,D,F) in embryos fixed 6-7 hours after electroporation with pCX-*LSox5*. A few transformed cells ectopically expressing LSox5 can be detected scattered within the neural tube with the anti-LSox5 serum (A,B). In situ hybridisation for *RhoB* at a mesencephalic level shows that the ectopic activation of this gene in cells at the electroporated side is analogous to the expression of LSox5 (C). The cells that ectopically express *RhoB* correspond to those with higher levels of ectopic LSox5 expression in their nuclei (D-F).

F). Interestingly, this induction was transitory in intermediate and ventral regions of the neural tube because 12 hours after electroporation only a fraction of the EGFP-labelled cells here expressed *RhoB*. Similarly, after 20 hours, the dorsal domain of *RhoB* expression had expanded and a few scattered ectopic *RhoB*-expressing cells were detected out of this region.

LSox5 misexpression upregulates the expression of *FoxD3* and *Sox10*

The transcription factors Sox10 and FoxD3 have both been implicated in the generation of the neural crest and in the posterior selection of crest-derived non-neural phenotypes (Aoki et al., 2003; Britsch et al., 2001; Dottori et al., 2001; Kos et al., 2001). Given the expression pattern of LSox5 in the peripheral glia, we examined whether *LSox5* misexpression influenced either *Sox10* or *FoxD3* activity. The expression pattern of these two factors did not appear to have been altered by the misexpression of *LSox5* within 6-7 hours of electroporation (data not shown). However, 12 hours after electroporation, the number of cells expressing both *Sox10* and *FoxD3* increased on the electroporated side of the embryo (Fig. 4A-D). Under these conditions, the embryos had developed to stage 11-11+ when the generation of the neural crest at mesencephalic and rostral hindbrain levels has normally terminated, and the neural crest cells are already at a distance from the dorsal neural tube. Indeed, this distancing of the neural crest population was apparent on the control side of the embryos. By contrast, cells continued to delaminate, and *Sox10*- and *FoxD3*-expressing cells were still detected in the neural tube on the electroporated side (Fig. 4B,D). This result indicates that the capacity to produce neural crest cells was temporally extended following *LSox5* misexpression.

Similar, yet more dramatic, phenotypes were observed 20-24 hours after electroporation, when the embryos had reached stages 13-14 (Fig. 4E-K). In these embryos, retarded migratory cells expressing *Sox10* and *FoxD3* were found in the midbrain and rostral hindbrain, where neural crest production normally ceases around stage 11 (Fig. 4E,G,K). In more caudal regions, *Sox10* and *FoxD3* expression was significantly augmented in the different migratory streams of crest cells, particularly in the circumpharyngeal crest (Fig. 4H-K). Cells exiting the neural tube at aberrant, more ventral locations were also observed in these embryos (Fig. 4L,M). Moreover, as for *Slug*, the ectopic cells expressing *LSox5* did not express *Sox10* or *FoxD3* (Fig. 4L,M).

LSox5 misexpression drives neural crest differentiation towards non-neuronal phenotypes

The persistence of LSox5 in the cephalic neural crest derived glial lineage, and the increased expression of both *Sox10* and *FoxD3* upon LSox5 misexpression, prompted us to analyse the fate of electroporated cells. In embryos that were allowed to develop for 48 hours after LSox5 electroporation, many EGFP-labelled cells reached the branchial arches or were spread across the most rostral mesenchyme (not shown). Thus, it seemed likely that these cells might be related to the non-neural crest derivatives. In the cranial ganglia, electroporated cells were preferentially detected at sites where Schwann cell precursors accumulate, both at the entrance and exit of the corresponding cranial nerve (Fig. 5). We also found labelled cells interspersed with neuronal precursors of placodal origin at the distal region of the trigeminal ganglion (Fig. 5A,B), the geniculate ganglion (Fig. 5C,D) and the petrosal ganglion (not shown). By contrast, labelled cells were very rarely found in the proximal region of the trigeminal ganglion where neural crest-derived neuronal precursors accumulate. In the ciliary and superior ganglia, the neuronal precursors are all of neural crest origin, but they did not coincide with LSox5 overexpressing cells (Fig. 5E-H). These data suggest that LSox5 overexpression in neural crest cells drives the differentiation of non-neuronal phenotypes.

SSox5 behaves as LSox5 when misexpressed in the neural tube

As well as LSox5, a shorter isoform is also generated from the *SOX5* genes, SSox5 (Fig. 6A), which contains only the HMG box for DNA binding and a unique coiled-coil domain (Denny et al., 1992; Wunderle et al., 1996). Although SSox5 is expressed in adult testis in the mouse (Hiraoka et al., 1998), it was not detected in embryonic chick extracts. However, as a first approach to analyse the functional domains responsible for the activity of LSox5, we analysed the effects of expressing the putative chick orthologue of mammalian SSox5 in the neural tube. With respect to the expression of *RhoB*, *Sox10* and *FoxD3* after electroporation, this short form behaved very similarly to the long isoform (Fig. 6B,C). These results indicate that the structural information contained in SSox5 is sufficient to

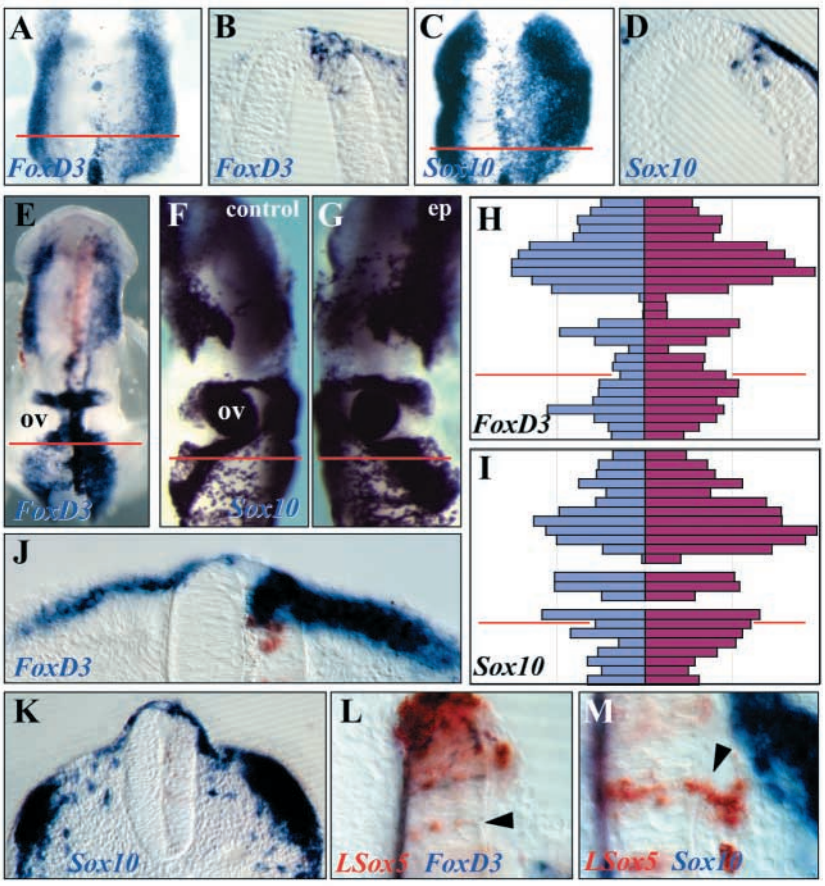

Fig. 4. Ectopic *LSox5* expression in the cephalic neural tube upregulates the expression of specific neural crest markers. Embryos were electroporated with pCX-*LSox5* on the right-hand side of the neural tube, left to develop for 12 (A-D) or 20-24 hours (E-M), and then subjected to in situ hybridisation to visualise the expression of *LSox5* and/or *FoxD3* or *Sox10*. (A,C) Dorsal view of the midbrain and anterior hindbrain of two embryos showing an increase in *FoxD3*- and *Sox10*-expressing cells on the transfected side. Transverse sections of these embryos (B,D) show the appearance of ectopic premigratory cells expressing these markers. Dorsal (E) or lateral views (F,G) of two treated embryos show the dramatic increase in *FoxD3* and *Sox10* expression on the transfected side (ep) 20-24 hours after electroporation. The effect is particularly remarkable in transverse sections at the level of the circumpharyngeal crest (J,K). (H,I) Diagrams representing the area covered by *FoxD3*- or *Sox10*-expressing cells in 40 μm serial sections through the cephalic region of the same embryos. These data enable us to estimate the number of cells expressing these markers along the anteroposterior axis (in arbitrary units). A larger area of expression is associated with the transfected side (red bars) in most sections. The red lines correspond to the transverse section in B (A), D (C), J (E,H) and K (F,G,I). (L-M) Transverse sections at a mesencephalic level showing transformed neuroepithelial cells that seem to leave the neural tube beyond the dorsal competence domain (arrowheads) and do not express *FoxD3* or *Sox10*. ov, otic vesicle.

influence neural crest development in the manner that we have observed with LSox5.

Discussion

Structural features of chicken LSox5

We have cloned the chicken orthologue of the *Sox5* gene, which appears to generate at least two long isoforms (LSox5-I and -II) during early embryogenesis. *Sox5* is a member of the D subgroup of the Sox gene family (Schepers et al., 2002). A short isoform of Sox5 (SSox5), containing an HMG DNA-binding domain and a small coiled-coil region, was originally described in adult mouse testis (Denny et al., 1992). Long isoforms were later found in mammals (Hiraoka et al., 1998; Lefebvre et al., 1998; Ikeda et al., 2002) that, like other members of the D subgroup (Sox6, Sox13), contain an extra coiled-coil domain and glutamine-rich stretches. In spite of the tight regulation that restricts SSox5 expression to specific adult tissues, we have shown that SSox5 is capable of producing the effects characterised in this study. If LSox5 does not harbour a transactivation domain, as suggested in some in vitro studies (Lefebvre et al., 1998), the formation of heterodimers with other transcription factors should be considered and the unique

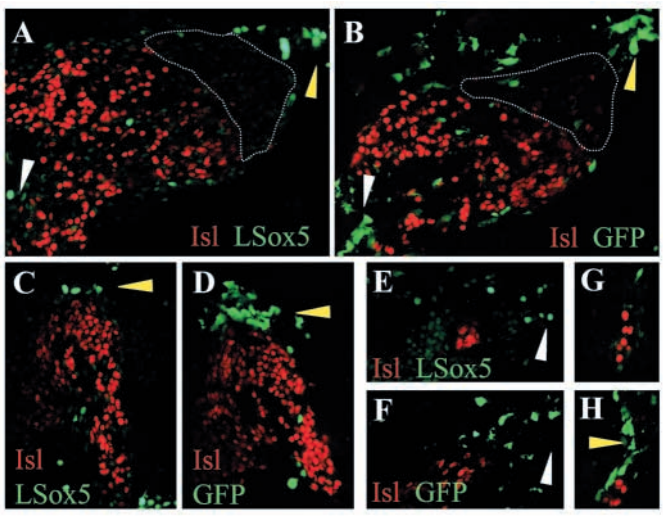

Fig. 5. Deviation of neural crest derivatives to the glial lineage. Parasagittal sections of the head region of embryos 48 hours after electroporation of *LSox5* showing the formation of the trigeminal (A,B), facial (C,D), ciliary (E,F), and superior (G,H) ganglia. Serial sections immunostained for LSox5 (green in A,C,E,G) or EGFP (green in B,D,F,H) show the distribution of transformed neural crest cells that maintain LSox5 overexpression. The exposure time was reduced in A,C,E,G to visualise only LSox5-overexpressing cells with signals above the normal level in glial cells. These cells are preferentially located at the proximal entrance of the corresponding cranial nerve (yellow arrowheads) or at the distal exit point (white arrowheads). Transformed cells are excluded from the proximal region of the trigeminal ganglion (broken line in A,B), where neural crest derived neurons should differentiate, and there is a consistent failure to colocalise with the neuronal marker (Islet, in red).

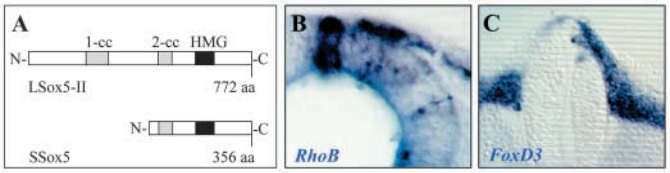

Fig. 6. SSox5 mimics the effects of LSox5 in misexpression assays. (A) Comparison of the LSox5-II and SSox5 polypeptides. HMG, HMG binding domain; cc, potential coiled-coil domains. (B,C) Transverse sections at rostral hindbrain levels hybridised with *RhoB* or *FoxD3* 12 hours after electroporation with pCX-*SSox5*. Both the ectopic expression of *RhoB* and the upregulation of *FoxD3* observed here are very similar to those produced after LSox5 misexpression (Figs 3-5).

coiled-coil structure present in SSox5 may represent an interactive domain.

Not only does the overall sequence of chick LSox5 show a high degree of similarity to that of its mammalian orthologue but the HMG box is identical. Nevertheless, one structural feature that distinguishes chick LSox5 derives from the fact that 16 out of the 33 amino acid substitutions between the mouse and the predicted chicken protein lie within residues 426-456. Strikingly, nine of these changes imply the gain of serine or threonine residues in the chick protein, including a specific serine rich domain (SRD). Indeed, when scanned for putative phosphorylation sites (Blom et al., 1999), they mainly lie in this region of LSox5 and some of the substitutions in the chick sequence generate new sites. This suggests that in the chicken, specific mechanisms may exist to regulate LSox5 activity by phosphorylation. Indeed, the regulation of Sox function by serine phosphorylation has been demonstrated: LSox5, Sox6 and Sox9 cooperate in the transactivation of a *Col2a1* chondrocyte-specific enhancer during cartilage formation, and the activity of Sox9 in this process is controlled by cyclic AMP-dependent protein kinase A phosphorylation (Huang et al., 2000).

LSox5 expression in the neural crest

We isolated chicken *LSox5* while searching for genes that are induced or upregulated between embryonic stages 8-11, a period during which the cephalic neural crest forms and begins to migrate. *LSox5* satisfied the criteria of the screen because it is expressed in the cephalic neural crest, both premigratory and migratory. However, we also detected *LSox5* expression in the trunk neural crest, albeit at much lower levels (not shown). At early developmental stages, *LSox5* is observed in the neural folds, but while it is expressed in the whole of the prospective forebrain epithelium, in more caudal regions it is restricted to the most dorsal part of the neural tube where the neural crest form. When compared with other neural crest markers, this expression pattern is unique to *LSox5*. The two genes *Slug* and *Sox9* are restricted to the dorsal region of the neural plate and the onset of their expression coincides with the generation of the neural crest. By contrast, *FoxD3* is first expressed in the most rostral region of the neural folds and only becomes restricted to the territories where the neural crest forms prior to delamination (Kos et al., 2002). Thus, the initial expression of *FoxD3* and the persistence of *LSox5* in the anterior prosencephalon are likely to be related to developmental processes other than the generation of the neural crest.

In the head, the onset of *LSox5* expression follows the well-established anteroposterior developmental gradient in the dorsal neural folds/tube, as do most other neural crest markers. However, *Sox9*, *Slug* and *FoxD3* all precede *LSox5*, which approximately coincides with *RhoB* and precedes *Sox10* expression. It is interesting to note that *LSox5*, as *Slug*, is continuously expressed at low levels throughout rhombomere (r) 3, while other markers such as *Sox9, FoxD3, RhoB* and *Sox10* are never expressed in this rhombomere (Liu and Jessell, 1998; Cheng et al., 2000; Kos et al., 2002) (S.P.-A., M.A.N. and J.A.B., unpublished). Although we do not know the functional significance of this at present, the absence of many neural crest markers may be related to the fact that the neural crest produced in r3 undergoes massive apoptosis (Graham et al., 1993). Those crest cells that survive in r3 are deviated rostrally or caudally to contribute to the major migratory streams adjacent to r2 and r4 (Birgbauer et al., 1995). Thus, based on their relative expression patterns, we would locate *LSox5* downstream of *Sox9*, *Slug* and *FoxD3*, and at the same hierarchical level or slightly upstream of *RhoB* and *Sox10*.

Early upregulation of *RhoB* caused by *LSox5* misexpression

Taking into account the gene hierarchy that emerged by analysing the endogenous expression in the neural crest, the upregulation of *RhoB* appears to be an immediate consequence of *LSox5* misexpression in the neural epithelium. This upregulation is a cell-autonomous phenomenon and it initially presents near complete penetrance, i.e. most transformed cells become *RhoB*-positive regardless of their location in the dorsoventral axis of the neural tube. With time, and in parallel with the number of *LSox5*-expressing cells within the neural epithelium, the number of *RhoB*-expressing cells increases dramatically at the dorsal aspect of the neural tube, concomitant with a massive delamination of cells expressing different neural crest markers. By contrast, the number of *RhoB*-expressing cells remains low at intermediate and ventral positions, suggesting that these environments are hostile to its induction. RhoB has been proposed to contribute to the changes in cell shape and adhesion that occur during the delamination of neural crest cells by regulating actin polymerisation, the formation of focal adhesions and of stress fibres (Liu and Jessell, 1998). Accordingly, it should be noted that some of the cells that are induced to express *RhoB* but that lie outside of the dorsal domain of neural crest competence, remain in the neural tube and maintain an epithelial aspect. Only a few cells located outside of, but close to, the domain of competence for crest formation seem to be able to delaminate and enter the adjacent mesenchyme. However, even when they are able to delaminate, these cells do not express any neural crest markers other than the ectopic *LSox5*. These results indicate that LSox5 is likely to induce *RhoB* expression directly. Moreover, although this makes the cells competent to undergo changes in cell shape, it is not compatible with them undergoing complete EMT and fully acquiring the neural crest phenotype, unless they are located in the crest-competent domain. We also describe the appearance of HNK1-positive cells within the neural epithelium as a consequence of *LSox5* misexpression, a phenotype similar to that described following

FoxD3 or *Sox9* misexpression (Kos et al., 2002; Cheung and Briscoe, 2003). Although we have not checked if this aberrant HNK1 expression coincides with or follows *RhoB* ectopic expression, it seems plausible that this phenomenon mimics the normal development of migratory neural crest cells, which acquire the HNK1 epitope after expressing *LSox5* and *RhoB*. In addition, although we do not know the molecular mechanisms involved in this particular behaviour, they may also operate under physiological conditions. Whereas physiological expression of Slug, Sox9 and FoxD3 is restricted to the neural crest competent region in the dorsal neural tube, both RhoB- and HNK1-positive cells can be found at more ventral locations later in development (S.P.-A., M.A.N. and J.A.B., unpublished).

LSox5 misexpression increases neural crest generation

We have shown that *LSox5* misexpression in the neuroepithelium produces a dramatic increase in the generation of neural crest cells, which express several neural crest markers, including Pax7, Slug, FoxD3, RhoB, Sox10 and HNK1. Within the head region, this effect can be detected at all levels of the AP axis. Quantitative variation was found between embryos that might reflect the precise timing of the electroporation and analysis, and the efficiency of transfection. Under our standard electroporation conditions, the extension of the 'temporal window' of neural crest generation was best visualised in the mesencephalic and rostral hindbrain regions. Other aspects such as the increase in the number of migratory cells were more evident in the circumpharyngeal streams. Whereas the induction of *RhoB* expression can be detected soon after the appearance of *LSox5*, the other markers are only induced after longer periods of time, suggesting that their induction is indirect. Before delamination, the ectopic crest cells are located within a crest competent domain as defined by overexpressing *Slug* (del Barrio and Nieto, 2002). The expansion of this presumptive neural crest territory is paralleled by the extension of the region in which the basement membrane disassembles, allowing the delamination of the neural crest cells that follow the normal migratory pathways. In addition, the 'temporal window' of neural crest generation is also extended, as has been observed after the misexpression of other neural crest markers, such as *Noelin 1* (Barenbaum et al., 2000), *FoxD3* (Kos et al., 2001; Dottori et al., 2001), *Slug* (del Barrio and Nieto, 2002), *Sox10* (Aoki et al., 2003) and *Sox9* (Cheung and Briscoe, 2003). As a matter of fact, many features related to the increase of neural crest production after *LSox5* overexpression are similar to those described in the aforementioned studies. It is thought that neural crest overproduction occurs at the expense of other CNS cell types (Dottori et al., 2001; Cheung and Briscoe, 2003). Consequently, the overproduction of neural crest cells frequently appears to deplete the cells from the most dorsal region of the neural tube, as evident morphologically following *LSox5* misexpression.

Regardless of the generalised phenotype of neural crest overproduction reported here, it is of interest to place *LSox5* in the genetic cascade of neural crest development. The endogenous expression patterns tentatively locate it downstream of *Sox9*, *Slug* and *FoxD3*, implying it acts after neural crest induction and the segregation of the crest from the other neuroepithelial cells. The results obtained after *LSox5* overexpression indicate that it lies upstream of *RhoB* and *Sox10*, compatible with studies indicating that *RhoB* is involved in the delamination process and *Sox10* in the survival and maintenance of the stem cell properties in the migratory population (Paratore et al., 2001; Kim et al., 2003; Mollaaghababa and Pavan, 2003). However, earlier markers were also upregulated when *LSox5* was ectopically expressed in neural crest cells at more extended times, suggesting that a feedback loop may be at work in vivo. A precedent comes from in vitro studies of EMT induction where a balanced cross-modulation of cell-cell adhesion molecules, cell-ECM adhesion molecules and cytoskeletal molecules can trigger and orchestrate EMT (Newgreen and Minichiello, 1995; Somasiri et al., 2001). Moreover, in a quail neural epithelial cell system in vitro, the pharmacological inhibition of protein kinase C immediately affects the cytoskeleton, provoking transformation into crest-like cells (Minichiello et al., 1999) and the upregulation of *Slug* and of *Sox10* expression (D. F. Newgreen, personal communication). In addition, a non-autonomous cell response may be induced by extracellular molecules (membrane proteins, soluble factors or matrix molecules). Indeed, many neural crest cells in the migratory streams on the transfected side did not express the EGFP marker, raising the possibility of secondary or indirect induction.

With respect to signalling molecules, previous studies have shown that signals emanating from the non-neural ectoderm and paraxial mesoderm, such as the BMPs, WNTs and FGFs, induce neural crest differentiation (Liem et al., 1995; Ikeya et al., 1997; LaBonne and Bronner-Fraser, 1998). However, the overproduction of neural crest caused by the misexpression of both *FoxD3* and *Sox9* has been placed downstream of these dorsalising pathways (Dottori et al., 2001; Cheung and Briscoe, 2003). The same seems to hold true for *LSox5* misexpression. We have verified that *Bmp4*, *Bmp7* and *Wnt1* expression remains unaltered in treated embryos, although it was sometimes diminished as a consequence of the depletion of neuroepithelial cells in the dorsal tube (S.P.-A., M.A.N. and J.A.B., unpublished).

During chondrocyte differentiation, Sox9 is required for the expression of LSox5 (Akiyama et al., 2002), which subsequently interacts cooperatively with Sox6 and Sox9 to activate the type II collagen gene and promote chondrogenesis (Lefebvre et al., 1998). We have analysed chick *Sox6* expression by in situ hybridisation, verifying that it is not expressed in either the dorsal neural tube or in early migratory crest (S.P.-A., M.A.N. and J.A.B., unpublished). In the dorsal neural tube, Sox9 activates a pathway leading to neural crest induction, in which Sox10 is later induced (Cheung and Briscoe, 2003). Sox10 expression is then maintained in the peripheral glial lineage (Britsch et al., 2001), where it regulates the expression of P_0 (Peirano et al., 2000). Thus, it is tempting to speculate that in neural crest development a similar interaction between SoxE and SoxD factors to that observed during chondroblast differentiation occurs. In this case, LSox5 would be the downstream a member of the Sox E group, Sox9, and would interact with Sox10, another E-group member during the premigratory stages and/or later on during the differentiation of the glial lineage. In addition, as our misexpression experiments suggest LSox5 may lie upstream of

Sox10, the expression of which is induced later to cooperate at stages of differentiation. Alternatively, Sox10 expression could be independent of LSox5, but may be upregulated or induced after forced LSox5 expression, in which case it would enter in the pathway of neural crest generation by mimicking the role of *Sox9*.

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Table 1. Chicken *LSox5* exon-intron boundaries*

Exon number	Exon size (bp)	Splice acceptor	Splice donor	Start	End
IIa	232	ttccttccag GATGTCTTCC	CAACACAATG gtaggtttgc	62.526.687	62.526.918
IIb	127	ttccttccag GATGTCTTCC	TCCCTTGCAT gtgagtttc	62.526.687	62.526.813
III	211	cttctttcag GAAGTTGATG	GAACCAGAAG gtaaggcctg	62.561.475	62.561.685
IV	87	cttcactcag AAACTCCCAG	GAAATAAAAG gtaacgctct	62.621.206	62.621.292
V	173	tgatgcccag GGACTCCAGA	ACAGGAACAG gtgagtgatt	62.634.657	62.634.829
VI	69	ttctccacag ATTGCAAGAC	ACAGATCCAG gtcagagact	62.637.330	62.637.398
VII	121	tgttcagcag GTCCAGGGTC	GCGGGATGCA gtaagtcctg	62.681.278	62.681.438
VIII	86	tcttacttag GTGACCCCTA	CCAACTGCAG gtaagtggca	62.694.300	62.694.385
IX	147	gcttttacag CAGTTGTATG	CAAAAGCAAG gtactgtacc	62.723.455	62.723.601
X	178	ctcatcgtag GATGAAGTGG	AACACAATAG gtaagatcac	62.745.501	62.745.678
XIa	173	ttttttaag ATATCCTTTC	GACAGAAAAG gtcagtggct	62.751.433	62.751.605
XIb†	146	acgtcgtcag GTTATTTAAA	GACAGAAAAG gtcagtggct	62.751.433	62.751.605
XII	109	ccccatgtag GACAAAACGA	GATTCTGATG gtcagtacaa	62.766.837	62.766.945
XIII	174	ctctttttag GAAGTGCAGG	AAGATACTAG gtaagtttgg	62.767.803	62.767.975
XIV	217	tttgtggcag GATCTCGCTG	TTAATGTTGG gtatgggacc	62.774.340	62.774.556
XV	-	tccattgcag GCAACAAGCA	–	62.777.731	-

*Exon-intron boundaries were identified by comparison of the chicken *LSox*5 cDNA sequence with the preliminary data set based on the first draft chicken genome assembly available in the Ensembl Genome Browser (http://www.ensembl.org). Ten bases on either side of the exon-intron boundary are shown for each junction. Exonic sequences are indicated by capital letters, with the splice acceptor and splice donor sequences shown in lowercase. Numbers in the last two columns indicate the position of the first and last base of each exon in chromosome 1.

†Alternative splice acceptor site deduced after analysis of the BBSRC ChickEST Database (http://www.chick.umist.ac.uk).