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Overexpression of Snail family members highlights their ability to promote chick neural crest formation

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SUMMARY

The Snail gene family of transcription factors plays crucial roles in different morphogenetic processes during the development of vertebrate and invertebrate embryos. In previous studies of function interference for one of the family members, *Slug*, we showed its involvement and neural crest formation in the chick embryo. Now we have carried out a series of gain-of-function experiments in which we show that *Slug* overexpression in the neural tube of the chick embryo induces an increase in neural crest production. The analysis of electroporated embryos shows that *Slug* can induce the expression of *rhoB* and an increase in the number of HNK-1-positive migratory cells, indicating that it lies upstream of them in the genetic cascade of neural crest development. The increase in neural crest production after *Slug* overexpression was confined to the cranial region, indicating that the mechanisms of crest induction somehow differ between head and trunk.

The expression of the two vertebrate family members, *Slug* and *Snail*, is peculiar with respect to the neural crest. *Slug* is not expressed in the premigratory crest in the mouse, whereas it is expressed in this cell population in the chick and the opposite is true for *Snail* (Sefton, M., Sánchez, S. and Nieto M. A. (1998) *Development* 125, 3111-3121). This raises the question of whether they can be functionally equivalent. To test this hypothesis both intra- and interspecies, we have performed a series of ectopic expression experiments by electroporating chick and mouse *Snail* in the chick embryo hindbrain. We observe that both genes elicit the same responses in the neural tube. Our results indicate that they can be functionally equivalent, although the embryos show a higher response to the endogenous gene, chick *Slug*.

Key words: Neural crest, Slug, Snail, RhoB, Chick, Functional equivalence, Epithelial-mesenchymal transition, DiI labelling, In ovo electroporation

INTRODUCTION

The activity of members of the *Snail* family of zinc-finger transcription factors is required in various developmental processes, in both vertebrate and invertebrate embryos. These factors have an evolutionarily conserved function in mesoderm development, in neural differentiation and in vertebrate neural crest formation. Additionally, they are involved in the determination of left-right asymmetry, in the process of endoreplication and recently, they have also been implicated in asymmetric cell division in *Drosophila* (for a review, see Hemavathy et al., 2000; Cai et al., 2001). Its function in mesoderm and neural crest delamination is known to be mediated by the triggering of epithelial-mesenchymal transitions (EMT), a process by which an epithelial cell is converted to a mesenchymal cell able to delaminate from an epithelium and to migrate through the extracellular matrix (Hay, 1995; Duband et al., 1995). The conserved role in mesoderm formation and the co-option of Snail/Slug to trigger EMT during crest delamination and the acquisition of the invasive phenotype in tumours (Cano et al., 2000) has led us to suggest that the triggering of EMT is a function specifically linked to the Snail gene family throughout evolution (Manzanares et al., 2001).

The first indication that this gene family was involved in EMT came from our previous studies of one of the family members in the chick embryo. Incubation of early chick blastoderms with antisense oligonucleotides to S*lug*, inhibited neural crest and mesoderm delamination from the neural tube and the early primitive streak, respectively (Nieto et al., 1994). Subsequently, defects in crest migration and lack of specific derivatives were demonstrated in the neural crest of *Xenopus* embryos after *Slug* antisense treatment (Carl et al., 1999). In addition, LaBonne and Bronner-Fraser (LaBonne and Bronner-Fraser, 2000) showed that *Slug* was necessary for both the formation of neural crest precursors and for neural crest migration (LaBonne and Bronner-Fraser, 2000). However, work carried out in the spinal cord of the chick embryo indicated that inhibition of neural crest delamination could occur in the presence of *Slug* (Sela-Donenfeld and Kalcheim, 1999). Since the majority of studies of crest delamination in relation to *Slug* have been carried out in the head region (Nieto et al., 1994; Carl et al., 1999; LaBonne and Bronner-Fraser, 2000), it cannot be excluded that different

mechanisms may operate for neural crest delamination in the head and the trunk.

Another putative caveat to the idea of Slug being crucial for EMT came from the phenotype of *Slug* null mutant mice (Jiang et al., 1998) that do not show defects in mesoderm or neural crest development. However, *Slug* is not expressed in the premigratory populations in the mouse since there is a very striking interchange in the expression patterns of the two family members (*Slug* and *Snail*) between chicken and mouse. This led to the suggestion that *Snail* rather than *Slug* could be the gene involved in EMT in the mouse (Sefton et al., 1998; Jiang et al., 1998). Indeed, we and others have recently shown that *Snail* triggers EMT in mammalian cells, acting as a repressor of the epithelial phenotype (Cano et al., 2000; Batlle et al., 2000). This raises the question of whether Snail and Slug can be functionally equivalent when ectopically expressed at the appropriate sites.

Therefore, we have decided to directly assess: (i) the role of Slug in chick neural crest development by overexpressing chick Slug all along the anteroposterior axis of the embryo, and (ii) the possibility of functional equivalence by ectopically expressing chick and mouse Snail in the chick hindbrain. By adopting this approach, we hoped not only to complement the previous function interference studies, but also to help clarify the role of this family in the formation of the neural crest.

Here we show that *Slug* is able to induce the formation of neural crest cell in the chick embryo. Moreover, we observed differences between the mechanisms involved in the formation of the neural crest in the head and in the trunk regions. Whereas in the cranial region *Slug* overexpression increases the number of premigratory and migratory neural crest, in the spinal cord *Slug* is only able to increase the number of crest precursors within the dorsal neural tube. This might have evolutionary implications with respect to the appearance of the neural crest and the role of the Snail gene family in this process. In relation to this, and based on ectopic expression studies of chick and mouse *Snail* in the chick hindbrain, we show that neural crest formation is a conserved role associated with the Snail family of zinc-finger transcription factors.

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).

In ovo electroporation and DiI labelling

After incubation to obtain embryos at stages 9-12, the eggs were windowed on one side. After visualising the embryo, a solution containing full length chick Slug, chick Snail or mouse Snail cDNAs cloned in pCDNA3 (Invitrogen) (5 mg/ml) was injected into the lumen of the neural tube in the head (stage 9) or in the trunk region (stage 11-12) together with GFP cDNA cloned in EGFPN1 (Clontech, 1 mg/ml). In ovo electroporation (Itasaki et al., 1999) into the right hand side of the neural epithelium was carried out with an Intracept TSS10 pulse stimulator (Intracell) using 5, 50 msececonds, 30 V pulses. In all experiments, the control side was to the left. In some cases, a DiI solution in 10% ethanol (Molecular Probes, C-7000) was also injected into the lumen of the neural tube just after electroporation. The eggs were sealed and allowed to develop for a further 15-30 hours. At the required stages, embryos were photographed in ovo with a Leica MZFLIII dissecting microscope to record GFP expression and DiI labelling. Subsequently, they were dissected and fixed overnight in 4% paraformaldehyde in PBS at 4°C. After fixation, a fraction of the DiI-labelled embryos were washed in PBS and sectioned in a vibratome to obtain serial 50 μm slices that were photographed and the area occupied by DiI-labelled migratory cells was quantified for the entire hindbrain region in the control and electroporated sides using the Analytical Imaging Station (AIS, from Imaging). Within the DiI-labelled area outside of the neural tube, only the surface showing fluorescence above background levels was considered. For the pictures shown in Fig. 1F-H, sections were examined to detect either DiI or GFP and then photographed using a double-exposure to get a merged image using a Leica DMR microscope and a conventional camera. These embryos (n=34) were electroporated with a bicistronic plasmid containing both Slug and GFP cDNAs (ClonTech #61011). All the remaining embryos were subjected to in situ hybridisation and/or immunohistochemistry as described below.

In situ hybridisation and immunohistochemistry

Single or double labelling in situ hybridisation experiments were carried out by simultaneous hybridisation with two probes as described by Nieto et al. (Nieto et al., 1996). One probe was labelled with digoxigenin-UTP (Boehringer Mannheim) and the second with fluorescein-UTP (Boehringer Mannheim). Digoxigenin-labelled probes were synthesized from the full-length cDNA of chick *Slug* and from plasmids containing fragments of the *Pax3*, *rhoA*, *rhoB*, and *cadherin 6B* cDNAs (kindly provided by M. Bronner-Fraser, T. Jessell and M. Takeichi, respectively). Fluorescein probes were synthesised from a plasmid containing neomycin cDNA sequences to visualise electroporated cells by detection of transcribed plasmid sequences (kindly provided by D. Duboule). After hybridisation, the embryos were incubated with alkaline phosphatase-conjugated anti-digoxigenin and anti-fluorescein antibodies. The alkaline phosphatase activity was detected by incubation with NBT/BCIP for digoxigenin probes (blue) and INT/BCIP for fluorescein-labelled probes (red, both from Boehringer Mannheim). In some cases, the embryos were then subjected to immunohistochemistry with HNK-1 antibody (prepared from the cell line obtained from ATCC) as described by Nieto et al. (Nieto et al., 1996). Some embryos were directly processed for immunostaining with anti-laminin antibody (Sigma, L9393; 1:500).

Following hybridisation and/or immunohistochemistry, embryos were fixed in 4% paraformaldehyde in PBS, washed in PBS and photographed in whole mount under a Leica M10 dissecting scope. In the majority of cases they were washed in PBS containing 50% glycerol, flat mounted and photographed using a Leica DMR microscope with Nomarski optics with an Olympus DP-10 digital camera. Subsequently, they were again washed in PBS and 15 μm paraffin sections (Fibrowax, BDH) or 50 μm vibratome slices in gelatine (Sigma) were obtained.

Mock electroporated embryos with empty pcDNA3 were hybridised with all probes used in this study in order to rule out possible crosshybridisation between probe and vector sequences. No significant effects were observed in these control embryos.

RESULTS

Slug overexpression through in ovo electroporation increases the migratory cell population in the chick embryo hindbrain

We have used in ovo electroporation as a means of overexpressing chick *Slug* in the hindbrain of the chick embryo, to assess the effects that this may have on the development of the neural crest. *Slug* is normally expressed in

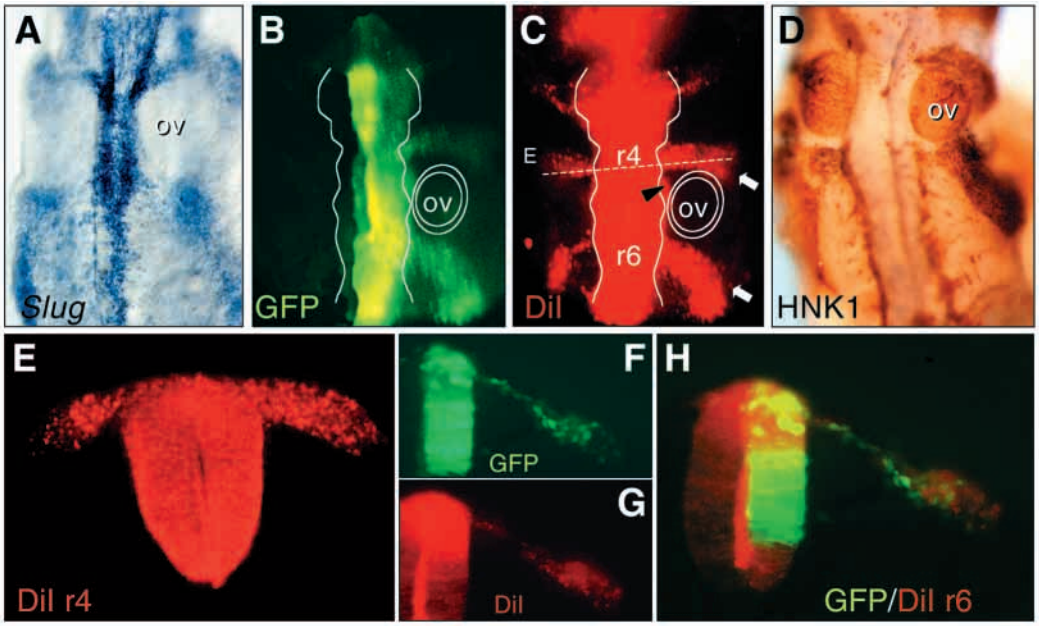

Fig. 1. Slug overexpression increases neural crest production in the chick hindbrain. (A) Stage 13 control embryo showing the normal *Slug* expression in the premigratory and migratory neural crest. (B-D) Embryos electroporated with plasmids containing chick Slug and GFP cDNAs, injected with DiI at stage 9 and analysed 15 hours later (stage 13-14). GFP expression is observed in the right hand side of the neural tube and in cells migrating from this side (B). DiI labelling is observed within the neural tube and in all crest cells that have emigrated from the neural tube after electroporation (C). (D) HNK-1 staining in the same embryo. Both DiI labelling and HNK-1 immunohistochemistry confirm the increase in neural crest production in the electroporated side. White arrows in C indicate the r4 and r6 crest streams where an increase in the migratory cell population can be observed when compared to the control side. Black arrowhead shows DiI-labelled cells adjacent to rhombomere 5 (see also Fig. 2H). A representative section taken at the level of r4 is shown in E to better assess the relative increase in crest production. (F-H) A section taken from a different embryo at the level of r6 showing (F) exogenous GFP-Slug expression, (G) DiI labelling and (H) the merged image. ov, otic vesicle; r, rhombomere. In all experiments, the control side is to the left.

the premigratory and migratory hindbrain crest populations as previously described (Nieto et al., 1994) and as can be observed in the flat mount of a stage 13 embryo shown in Fig. 1A. We have electroporated chick *Slug* together with *GFP* in the hindbrain of stage 9 chick embryos as described in Materials and Methods, and analysed the phenotype 15 hours later when they had reached stage 13-14. Migratory DiI- and/or GFP-labelled cells observed in these embryos correspond to neuroepithelial cells that have migrated from the neural tube after electroporation. As can be seen in the embryo shown in Fig. 1B, the majority of cells in the right hand side of the neural tube incorporated the plasmids and show GFP expression, in this case from the posterior part of rhombomere 2 to the border with the spinal cord. In addition, DiI labelling showed that in 88% of the embryos (66/75), there was a significant increase in neural crest migrating from the neural tube in the electroporated side (Fig. 1C, white arrows). HNK-1 immunohistochemistry confirmed an increase in the migratory neural crest population on the right hand flank of these embryos (Fig. 1D). In order to quantify the increase in the migratory population, we carried out a serial section analysis of some of the DiI-labelled embryos (n=7) by measuring the area occupied by DiI-labelled cells in the control and electroporated sides in the hindbrain region as described in Materials and Methods. Our results show a 208±37% of increase (mean±s.e.m.; n=7). One of the representative sections is shown in Fig. 1E. Fig. 1F-H show a section from a different DiI-labelled embryo that was electroporated with a plasmid containing both *Slug* and GFP. This section was taken at the level of r6. Note the overlap in the distribution of the GFP and Slug-expressing cells (Fig. 1F) with those that have emigrated from the neural tube after the electroporation process (Fig. 1G).

In a separate set of experiments, we looked for *Slug* expression in the electroporated embryos (n=13). Although we could not distinguish between the exogenous and the endogenous gene in the migratory population, we were interested in determining the amount of *Slug*-positive cells that resulted from the electroporation of the exogenous gene. Fig. 2A shows one of these embryos at the post-otic level. See the high level of *Slug* expression all along the right side of the hindbrain and the increased population of *Slug*-expressing cells migrating from it. This was observed in all the embryos analysed (n=13). Double labelling with HNK-1 confirmed that many of these migratory cells were double-positive (white arrowheads), although some ectopic cells located close to the right side of the tube and posterior to r6 were only *Slug* positive (Fig. 2A,B, black arrowheads). This can be better observed in the sections taken form this embryo and shown in Fig. 3A,B. See below for an analysis of different migratory crest populations. These results indicate that increased expression of Slug in the neural tube induces the formation of supernumerary crest cells in the hindbrain.

Slug also increases the premigratory cell population and induces the expression of r*hoB and Pax3* in the developing chick hindbrain

In order to analyse the phenotype of the electroporated embryos in more detail, the expression of *rhoB* was studied. This member of the family of small GTPases is expressed in the premigratory and early migratory crest and has been implicated in the delamination of the neural crest from the spinal cord (Liu and Jessell, 1998). In the control side (left) of stage 13 embryos, *rhoB* expression was detected in a small population of premigratory crest cells (compare Fig. 2C with the expression of *Slug* in Fig. 1A) and in early migratory cells delaminating from r4 and r6. However, in the

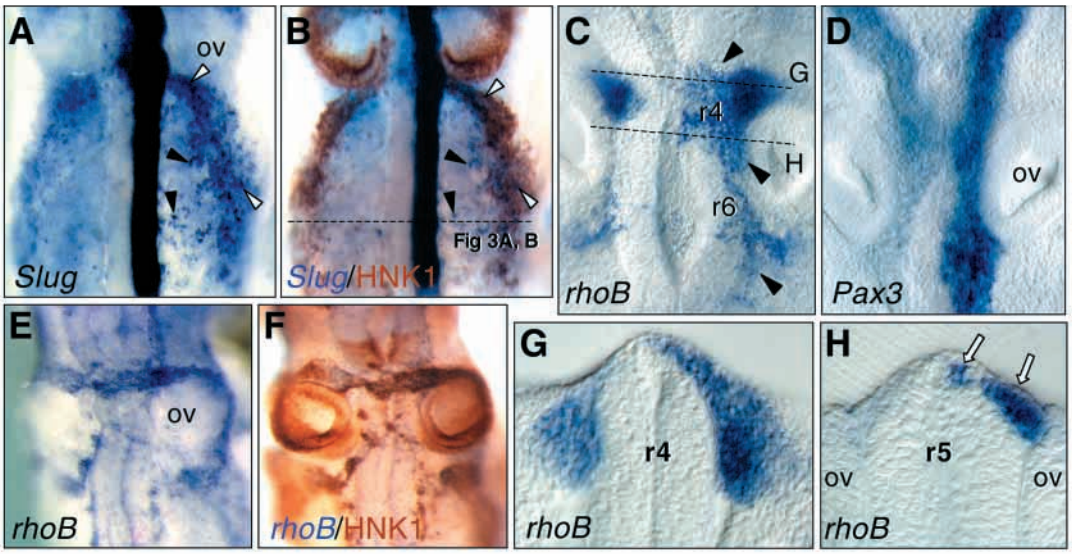

Fig. 2. Slug expression directly or indirectly induces the expression of *rhoB* and *Pax3* in the chick hindbrain. (A, B) Embryo electroporated with chick *Slug* at stage 9 and analysed 15 hours later (stage 13) by in situ hybridisation with chick *Slug* (A) and subsequently subjected to HNK-1 immunohistochemistry (B). The electroporated cells on the right hand side of the neural tube express high levels of Slug and more Slug-expressing cells can be observed migrating from the post-otic hindbrain. It is interesting to note that although many Slug-expressing migratory cells are also HNK-1 positive (white arrowheads in A and B), some migrating cells do not show HNK-1 reactivity (black arrowheads in A and B). The dotted line shows the level of the section in Fig. 3A. (C,D) Different embryos subjected to the same electroporation process and hybridised with *rhoB* or *Pax3* at stage 13. Note the ectopic *rhoB* expression in the premigratory crest of rhombomeres 4 and 5 and a greater area covered by the *rhoB*-expressing cells emigrating from r4 and r6. This can be better observed in the sections (G,H) taken from this embryo at the levels indicated by the dotted lines in (C). White arrows in H indicate cells migrating out from r5. (D) An increase in the levels of *Pax3* expression can be seen all along the AP axis of the hindbrain. (E,F) Another stage 15 embryo illustrating *rhoB* (E) and HNK-1 expression (F) after *Slug* electroporation. Note that there is an increase in crest cells migration from the right hand side, which expresses the two markers. Abbreviations as in Fig. 1.

electroporated side (right) a larger number of cells expressing *rhoB*, both premigratory and migratory, was detected (Fig. 2C). Vibratome sections at the level of r4 and r5 clearly show the increase in the number of *rhoB*-positive cells emigrating from the neural tube on the electroporated side (Fig. 2G,H).

It has been previously demonstrated that very few neural crest cells emigrate from r5. Moreover, those cells that do originate in r5 usually join the streams emigrating from r4 and r6 (Birgbauer et al., 1995; Graham et al., 1997). In electroporated embryos, *Slug* overexpression induced *rhoB* ectopic expression in the r5 neural epithelium and the accumulation of crest cells adjacent to it (Fig. 2H, white arrows). Similar phenotypes, in the r4 to r6 region were observed in 72% of the embryos (18/25). Double labelling with HNK-1 confirms that the majority of migratory *rhoB*-expressing cells also express this neural crest marker (Fig. 2E,F). We additionally analysed the expression of another member of the Rho family, *rhoA* (Liu and Jessell, 1998) also expressed in the neural tube at these stages, and we did not find any difference in the *Slug* electroporated embryos (n=7; not shown). These results suggest that *Slug* can specifically induce ectopic expression of rhoB in the chick hindbrain.

We have looked at *Pax3* expression because although it has been shown to precede *Slug* expression in the spinal cord, *Slug* is first to appear in the hindbrain (Buxton et al., 1997a). Fig. 2 D shows that *Slug* overexpression induces a higher level of *Pax3* expression in the hindbrain. However, this phenotype could be observed in only 30% of the embryos (6/20).

The Slug-induced increase in premigratory neural crest is restricted to dorsal territories

As we have shown above, *Slug* overexpression increases the production of crest cells in the hindbrain. The sections also indicate that supernumerary *rhoB-expressing* cells are only produced in the dorsal region of the tube. To better assess the dorsoventral phenotype in relation to the extension of the electroporated area along this axis we looked at *Slug*-expressing cells in sections taken from the *Slug* electroporated embryos. In Fig. 3A, which is a section taken from the embryo shown in Fig. 2B, clearly demonstrates that although there is ectopic *Slug* expression in the whole hemi-tube, crest cells migrate only from the dorsal part. As can be observed in the whole-mounted preparation (Fig. 2B), this double-labelled section shows that many of the cells that express *Slug* also express HNK-1, although there are ectopic cells close to the tube that only express *Slug* (Fig. 3A, Fig. 2B). A higher magnification photograph (Fig. 3B) shows the presence of heterogeneous populations of crest with respect to the expression of these two markers. Since HNK-1 expression is known to be acquired by crest cells after migration form the neural tube, we confirmed the existence of *Slug*-positive/HNK-1-negative cells located close to the neural tube not only in the control side but also in control embryos (Fig. 3C-E). Similar results were obtained for *rhoB*/HNK-1 double-labelled embryos at this stage (not shown).

The competence of cells at the different dorsoventral levels to respond to *Slug* was also confirmed in embryos hybridised with *rhoB* and *neomycin* probes (Fig. 4A). Detection of

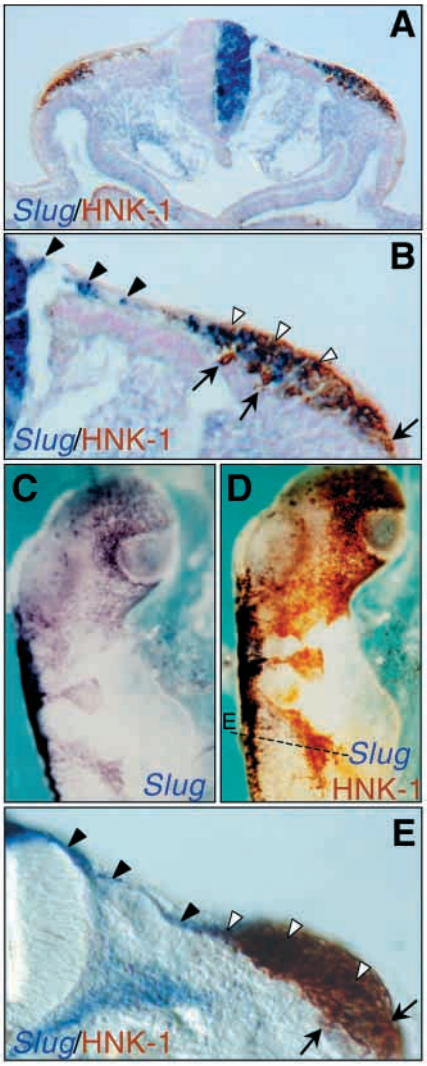

Fig. 3. Slug overexpression in the chick hindbrain increases the production of neural crest cells from the dorsal neural tube. (A) Embryo showing ectopic *Slug* expression at the right side of the hindbrain all along its dorsoventral axis. This section was taken from the embryo shown in Fig. 2B at the level indicated by the dotted line. Observe that *Slug*-expressing cells do not migrate from intermediate or ventral levels of the neural tube. A higher number of migratory cells can be observed in the electroporated side, some of which do not express HNK-1. This can be better observed in the magnification shown in B, where the existence of different migratory populations is readily apparent. Black arrowheads indicate *Slug*-positive/HNK-1-negative cells; white arrowheads exemplify the double-labelled population and black arrows indicate examples of *Slug*-negative/HNK-1-positive cells (see text). These populations are also detected both in the control side of the electroporated embryos (A) and in control embryos such as that shown in C-E, hybridised with *Slug* and subsequently stained with HNK-1. A section at the level of r6 is shown in E.

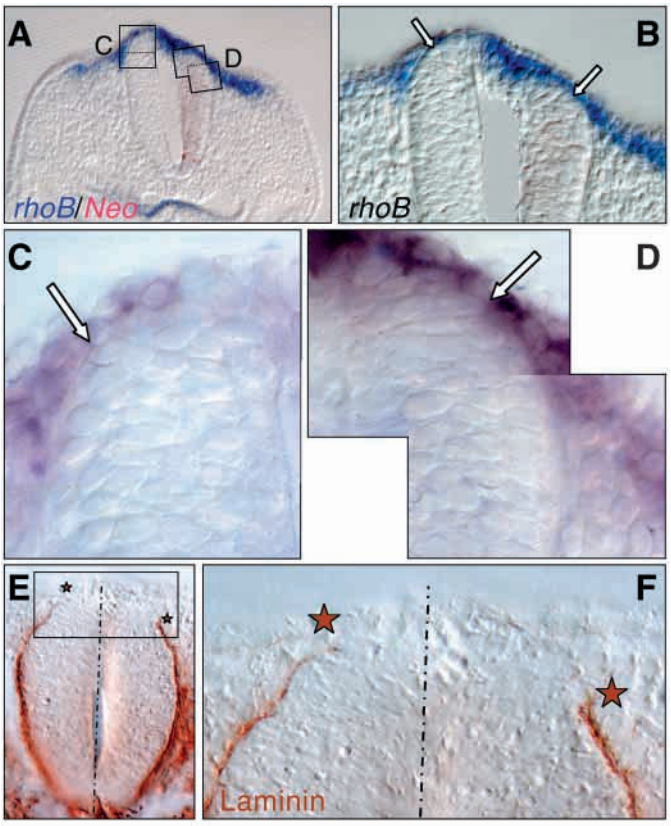

Fig. 4. Slug overexpression in the chick hindbrain induces ectopic epithelial-mesenchymal transition in the dorsal part of the neural tube. (A) A section from an embryo hybridised with *rhoB* (blue) and neomycin (red; to detect electroporated cells, see text). There is a clear increase in premigratory crest cells in the dorsal neural tube and a higher amount of *rhoB*-expressing migratory cells (B). (C,D) Composit images of high power views of the boxed regions in A, corresponding to control and electroporated sides, respectively. Observe the Slug-induced ectopic EMT concomitant with the breakdown of the basement membrane indicated by the white arrow in D. The site of breakdown is confirmed by immunostaining with the basement membrane marker laminin and indicated by stars in E and F.

neomycin enables all the electroporated cells to be identified although to avoid masking the signal obtained with other markers the visualisation of this probe was normally underdeveloped (pale red). On the side of the embryos that overexpressed *Slug*, the area of *rhoB*-expressing cells within the dorsal hindbrain was larger indicating that *Slug* is able to induce the formation of ectopic premigratory neural crest precursors in the dorsal neural tube (Fig. 4B). It has been proposed that *Slug* induces neural crest delamination by triggering EMT (Nieto et al., 1994; Nieto, 2001). A closer examination of this section (Fig. 4C,D) shows that indeed cells located in an extended region of the dorsal hindbrain are undergoing EMT. Note the difference in the position at which the basement membrane is undergoing breakdown on both sides of the neural tube (white arrows). This is confirmed by the detection of the basement membrane marker laminin in *Slug* electroporated embryos. A representative section of these embryos is shown in Fig. 4E,F.

The increase in neural crest migration induced by Slug is confined to the head region of the developing chick

Having seen that *Slug* overexpression increases the formation of neural crest in the chick hindbrain, we wanted to determine whether the same may occur in other regions along the

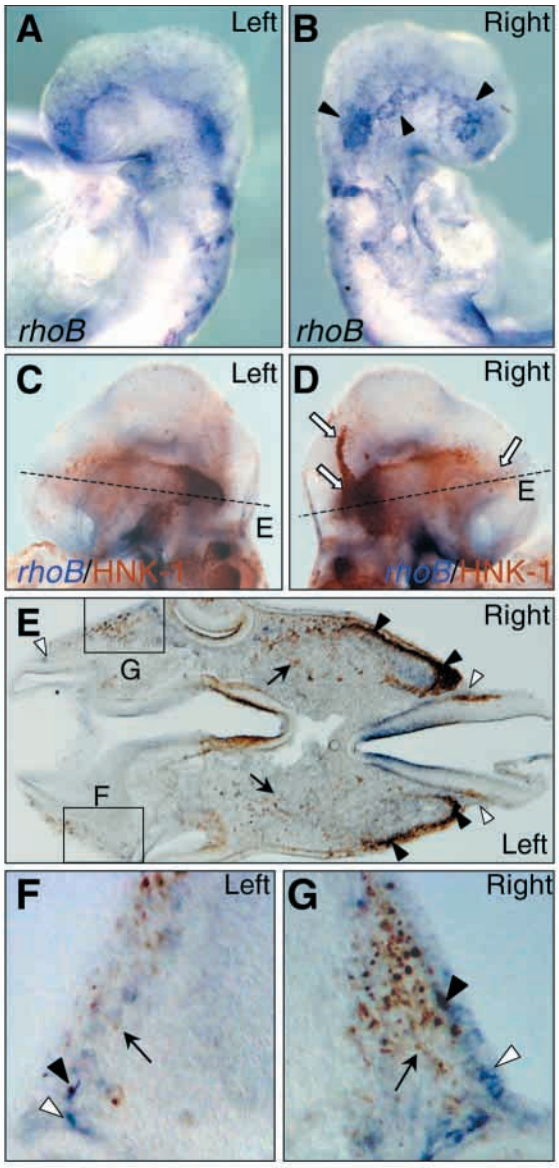

Fig. 5. Slug overexpression increases the migratory neural crest population in the cranial region of the chick. Control (A) and electroporated sides (B) of the same whole-mounted embryo hybridised with *rhoB* 24 hours after electroporation with *Slug* (stage 15). In B, observe the higher number of *rhoB* positive cells in the crest cells migrating from the forebrain, midbrain and hindbrain regions (arrowheads). (C,D) Another *Slug* electroporated embryo double labelled for *rhoB* and HNK-1 at stage 17. (E) A section taken from this embryo at the different levels indicated by the dotted lines in C and D allows the visualization of different crest populations that are increased in the electroporated side (top in this picture). (F,G) High power views of the boxed areas in E. Black arrowheads, *rhoB*-positive/HNK-1-positive cells; white arrowheads, *rhoB* positive cells; black arrows, HNK-1-positive cells.

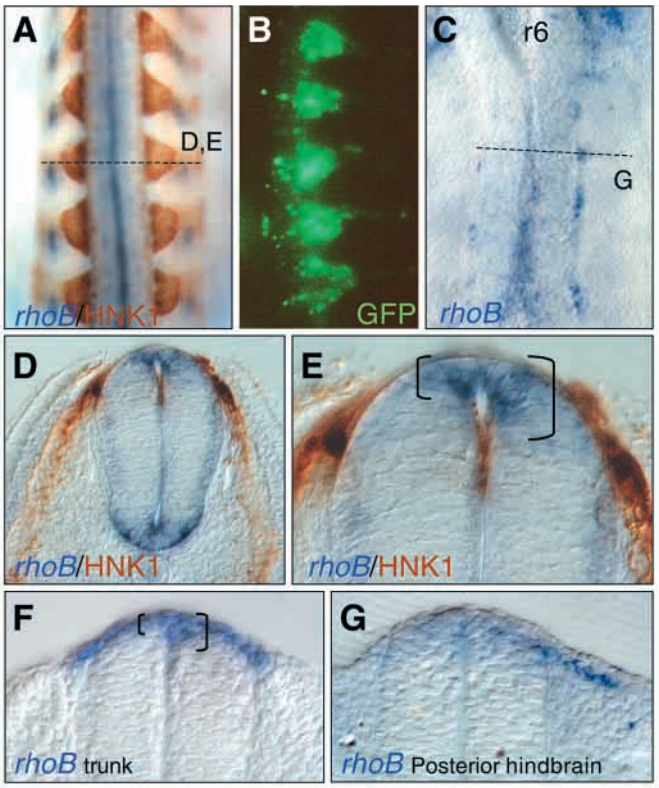

Fig. 6. Slug overexpression increases the premigratory neural crest population in the trunk. (A) Trunk level of an embryo processed for *rhoB* and HNK-1 expression 30 hours after being electroporated with *Slug* at stage 12. No differences can be detected in the migratory neural crest of control and experimental sides although numerous electroporated GFP-expressing cells had migrated from the tube and can be seen in a similar pattern to that observed for HNK-1 (B). (D,E) A section of this embryo, confirming that the migratory population is similar in both sides. However, note an increase in the *rhoB*-expressing area in the right half of the spinal cord (E, brackets). This is better observed in a similar embryo only hybridised with *rhoB* (F). (C) Flat mount of the posterior hindbrain of an embryo electroporated at stage 9. The increase in *rhoB*-expressing crest cells both in the premigratory and in the migratory population can be better assessed in the section taken at the level of the dotted line (G).

anteroposterior axis of the neural tube. We first analysed more anterior head regions by electroporating embryos at stage 9 from the midhindbrain to the prosencephalon and observed an increase in migratory crest cells in 77% of the embryos (7/9). One of these embryos analysed at stage 15 is shown in Fig. 5A, B. In the diencephalon, the midbrain and r4 of the hindbrain, an increase in the number of migratory *rhoB*-expressing cells was observed in the side of the embryo overexpressing *Slug* 24 hours after electroporation (Fig. 5A,B, arrowheads). Another embryo double labelled for *rhoB* and HNK-1 at stage 17 shows an increase in similar crest populations (Fig. 5C,D, also shown in a section Fig. 5E). Some of the *rhoB*-expressing cells were also labelled by HNK-1 (white arrowheads). Another population of cells express *rhoB* but not HNK-1. Both these cell populations were found on either side of the head, although a higher number were found on the electroporated side (black arrowheads). There is still a further population of cells that contain HNK-1 but that did not express *rhoB* (black arrows). The nature and amount of these populations are better observed in the control and in the electroporated side in the magnified views shown in Fig. 5F,G, corresponding to the boxed areas in Fig. 5E. Analysis of control embryos confirms their presence (not shown).

We also overexpressed *Slug* in the developing spinal cord by electroporating embryos at stage 11-12 and analysing the

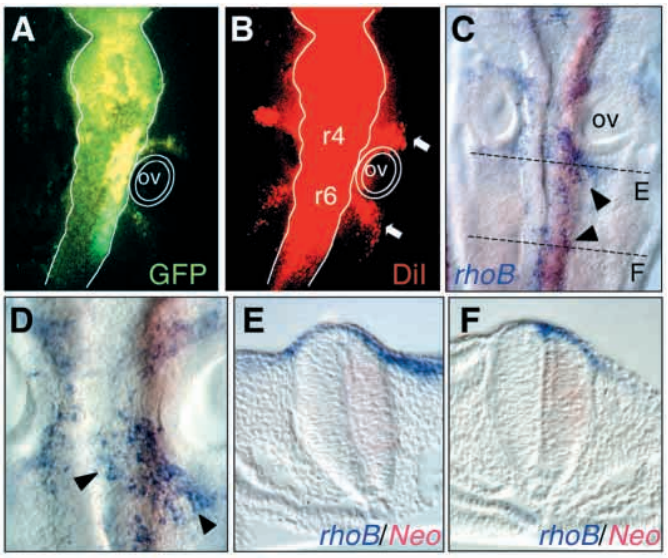

Fig. 7. Ectopic expression of chick Snail increases neural crest production in the chick hindbrain. Electroporations were carried out as described in the legend to Fig. 1. (A) GFP expression and (B) DiI labelling to visualise the neural crest cells that have emigrated from the hindbrain. C illustrates a similar embryo showing *rhoB* expression (blue). This embryo was also hybridised with a neomycin probe to detect the electroporated area (pale red). (D) A high power view showing the increase in premigratory and migratory neural crest (arrowheads). (E,F) Sections taken from the same embryo at the levels indicated by the dotted lines in C.

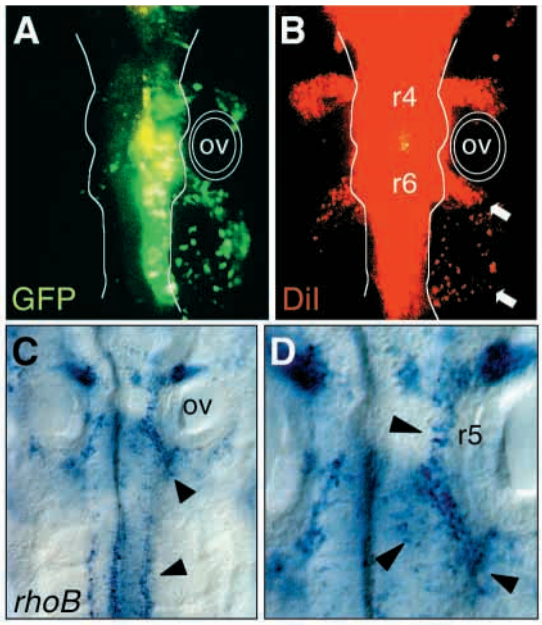

Fig. 8. Ectopic expression of mouse Snail also increases neural crest production in the chick hindbrain. Electroporations were carried out as described in the legend to Fig. 1. (A,B) GFP expression and DiI labelling identifying neural crest emigrating from the hindbrain. In C a similar embryo shows ectopic *rhoB* expression (blue). (D) A high power view of premigratory and migratory neural crest. Arrowheads indicate regions of ectopic expression. Note the amount of *rhoB*-positive cells at the level of r5.

corresponding phenotypes 24-30 hours later. After *rhoB* and HNK-1 double labelling, we could not observe differences in the amount or in the pathways taken by the migratory cells (n=15) (Fig. 6A). The amount of GFP-expressing cells migrating from these axial levels confirmed that the spinal cord had been extensively electroporated (Fig. 6B). In sections taken from these embryos, we confirmed the absence of differences in the migratory population of both sides (Fig. 6D). However, an increase in the area of *rhoB*-expressing premigratory crest cells could be detected in the electroporated side in 78% of embryos (7/9; Fig. 6E). This increase is better observed in *rhoB*-single labelled embryos (Fig. 6F). We also analysed the expression *cadherin6B*, a marker of premigratory neural crest cells whose expression precedes that of *Slug* in the neural tube. No significant differences were seen in the embryos analysed (n=11; not shown) as was the case for *Pax3* (n=15; not shown), which in the trunk, is also expressed before *Slug* (Buxton et al., 1997a).

After looking carefully at the postotic region of the electroporated embryos we observed an increase in premigratory and migratory *rhoB*-expressing cells up to the level around the fourth and fifth somites. This is shown both in the flat mount (Fig. 6C) and in a section taken from it (Fig. 6G). Interestingly, this axial level coincides with the border between the hindbrain and the spinal cord, as defined by chick-quail chimeras analyses (Cambronero and Puelles, 2000).

Ectopic expression of chick and mouse *Snail* increase neural crest production in the chick hindbrain

The swapping in expression of Slug and Snail in the regions of EMT has been described in chick and mouse. As far as the chick neural crest is concerned, *Slug* is expressed in both the premigratory and migratory populations whereas *Snail* is only present in a subpopulation of migratory cells at a distance from the neural tube (Sefton et al., 1998). Since the opposite is true in the mouse, we suggested that the role played by Slug in the chick might be performed by Snail in the mouse (Sefton et al., 1998). This is in keeping with the fact that Snail is able to convert mammalian epithelial cells into migratory and invasive mesenchymal cells (Cano et al., 2000; Batlle et al., 2000). This raises the question of whether the two vertebrate family members, Snail and Slug, can be functionally equivalent. To directly address this question, we have ectopically expressed chick Snail in the chick hindbrain following a similar protocol to that used in the experiments described above for chick *Slug* (Fig. 7). The electroporated embryos show a greater number of DiI-labelled cells (Fig. 7B) and a greater number of both premigratory and migratory and *rhoB*-expressing cells (Fig. 7C,D flat mount and E,F sections). The phenotype was milder than that observed with chick *Slug* and was readily visible in 59% (10/17) and 46% (6/13) of the embryos analysed for *Slug* and *rhoB*, respectively.

If *Slug* and *Snail* can be functionally equivalent in inducing the production of chick neural crest cells, we wanted to assess whether this equivalence could extend across species. To address this question, we electroporated mouse *Snail* into the chick hindbrain (Fig. 8). Mouse *Snail* induces a full EMT in murine and canine epithelial cell lines (Cano et al., 2000) and indeed, it was also able to increase neural crest production in the chick hindbrain. As in the case of chick *Snail*, the

phenotype observed was also milder than that obtained with chick *Slug*. Nevertheless, a high percentage of embryos (72%, 13/18) showed a significant increase in the number of DiI-labelled migratory cells (Fig. 8B) and in both premigratory and migratory *rhoB*-expressing cells in the electroporated side (54%, 7/13; Fig. 8C,D).

Taken together, these results indicate that vertebrate *Snail* family members are able to function as inducers of neural crest formation both intra- and interspecies, although the embryos exhibit stronger phenotypes after overexpression of the gene normally expressed in this tissue, chick *Slug*.

DISCUSSION

Slug overexpression increases neural crest production in the chick embryo hindbrain

Overexpression of chick *Slug* induced by in ovo electroporation in the hindbrain leads to an increase in the migration of chick neural crest cells. This can be clearly appreciated by the increase in the number of DiI-labelled cells that emigrate from the hindbrain and in the amount of cells that express the neural crest marker HNK-1 (Tucker et al., 1984). These results are in agreement with previous studies where interference with *Slug* function was shown to inhibit neural crest migration (Nieto et al., 1994). They are also in keeping with both loss- and gain-of-function experiments carried out in *Xenopus* embryos (Carl et al., 1999; LaBonne and Bronner-Fraser, 1998).

Looking at the overall pattern of migratory cells in the experimental side, it seems that although there is a high increase in this population, the overall migratory pattern is similar to that in the control side. The usual streams of cells that emerge from the even-numbered rhombomeres can be nicely appreciated, indicating that the normal migratory pathways are being followed. In these experiments, we have not assessed the contribution of individual rhombomeres but it is known that the number of crest cells that emigrate from r3 and r5 is small and that they join the streams of cells that migrate from r2 and r4 (Birgbauer et al., 1995). Nevertheless, crest-free areas arise through a combination of cell death (Graham et al., 1997) and the generation of exclusion zones adjacent to r3 and r5 (Farlie et al., 1999). These mechanisms seem to operate even in the presence of an excess of *Slug*, at least in the r3 region. However, upon closer examination of the embryos, a significant population of crest cells adjacent to r5 can be observed, whose movement is probably impeded by the presence of the otic vesicle (see Figs 1 and 2). They seem to be r5-derived crest although we cannot exclude the possibility of this population being r4 crest that accumulates in this region.

Since DiI labelling and HNK-1 staining do not provide information regarding the premigratory crest population, we examined the expression of premigratory crest markers. *Slug* expression was not useful because it detects all the electroporated cells. Neither was another marker of the premigratory population, *cadherin6B*, appropriate, since its expression is very transient and at the stages analysed, it is no longer expressed in the hindbrain (Nakagawa and Takeichi, 1995). In addition, the expression of *cadherin 6B* is more extensive and precedes that of *Slug* in the neural epithelium, making it unlikely that its expression would be affected by *Slug* overexpression. *RhoB*, a member of the Rho small GTPases family, is expressed in premigratory and early migratory crest cells (Liu and Jessell, 1998) and thus, expression of this gene was used to assess the effects that *Slug* overexpression had on crest precursors. In addition to detecting many more cells migrating from the hindbrain, we also observed an expansion in the area occupied by *rhoB*-expressing cells within the neural tube, indicating that *Slug* is involved in neural crest induction. Similarly, using an inducible inhibitory mutant LaBonne and Bronner-Fraser (LaBonne and Bronner-Fraser, 2000) demonstrated that Slug is implicated in both the formation of neural crest precursors and in neural crest migration in *Xenopus*. The expression of HNK-1 in the majority of the migratory crest population induced by *Slug* overexpression confirms their phenotype, although cells located at ectopic positions close to the neural tube are not labelled by HNK-1. This is compatible with HNK-1 being acquired in crest cells after their emigration from the neural tube upon receiving signals present in the migratory pathways.

The ectopic expression of *rhoB* in the *Slug* electroporated hindbrain indicates that *Slug* can directly or indirectly induce *rhoB* expression, which we can now consider a downstream target of *Slug*. This is not surprising, since *rhoB* has been implicated in crest delamination and in the sequential order of gene induction by BMP signalling it is preceded by *Slug* (Liu and Jessell, 1998). Thus, *Slug* is located upstream of *rhoB* in the temporal hierarchy of gene expression during neural crest formation.

Pax3 is another gene that is expressed very early in the neural tube, defining the dorsal regions including those that contribute to the formation of the neural crest (Goulding et al., 1993). Since *Slug* overexpression in turn elevated the levels of *Pax3* expression in the hindbrain, *Pax3* also appears to be another direct or indirect target of *Slug* in the hindbrain. This is compatible with the fact that the onset of *Pax3* expression follows that of *Slug* in the head region (Buxton et al., 1997a). Mutations in *PAX3* have been associated with Waardenburg syndrome (WS) (Tassabehji et al., 1992), as have mutations in *SOX10* and *MIFT*. Recently it has been suggested that SOX10, in synergy with PAX3, strongly activates MITF expression (Bondurand et al., 2000; Potterf et al., 2000). If we consider that chick *Slug* and *Sox10* show similar expression patterns and that *Slug* expression precedes that of *Sox10* (Cheng at al., 2000), it is tempting to speculate that *Slug* may be involved in melanocyte development by indirectly upregulating the expression of *Mift* through the activation of *Pax3* and possibly *Sox10*.

Since Snail family members are thought to act as repressors in animals ranging from *Drosophila* to human (Hemavathy et al., 2000), it is likely that *Slug* will only indirectly regulate the expression of both *rhoB* and *Pax3*, although the possibility of it acting as an activator cannot be excluded at the moment.

The competence of the hindbrain to respond to *Slug* overexpression is restricted to its dorsal territory

The ectopic *rhoB* expression induced by Slug does not extend to all the electroporated cells. Rather, and in a similar way to that observed in *Xenopus*, *Slug* overexpression increases the production of neural crest cells only in territories of endogenous expression or close to them (LaBonne and Bronner-Fraser, 1998). In our experiments, an expanded territory of *rhoB* expression is observed but it is, however, restricted to the dorsal part of the neural tube. This could be

partly explained by the fact that intermediate chick neural plate cells lose their capacity to form neural crest after stage 10 (Basch et al., 2000). Recently, the competence to become neural crest has been associated with the presence of noelin 1, a secreted glycoprotein present in the neural folds before Slug. Over-expression of noelin 1 in chick embryos leads to an increase in the period of crest production (Barembaum et al., 2000). Additional molecules such as targets of BMP, FGF of Wnt signalling that are not present in the ventral neural tube may also be required in conjunction with *Slug* (Liem et al., 1995; Ikeya et al., 1997; Mayor et al., 1997; Dorsky et al., 1998; LaBonne and Bronner-Fraser, 1998). Alternatively, or in addition, inhibition of *Slug* function by ventral signals can also operate in these embryos.

Slug overexpression induces ectopic epithelial-mesenchymal transition in the dorsal part of the chick hindbrain

Since inhibiting *Slug* activity in the early chick embryo impaired neural crest and early mesoderm delamination, it was originally suggested that *Slug* may trigger the process of EMT (Nieto et al., 1994). Further support for this hypothesis comes from the interpretation of experiments in which retinoic acid (RA) treatment induced the loss the mesenchymal phenotype in neural crest cells (Shankar et al., 1994). Since *Slug* is downregulated by RA (Ros et al., 1997; Buxton et al., 1997b; Romano and Runyan, 2000), it seems likely that the RA treatment was inhibiting Slug function. In this work, we demonstrate that *Slug* does indeed induce EMT in the chick hindbrain since the area covered by the cells that emigrate from the hindbrain is greater in regions where *Slug* is overexpressed. Moreover, the area of the neural tube that lacks basement membrane is also increased by this manipulation, the breakpoint of which can be seen more ventrally than on the control side.

Different mechanisms account for neural crest migration in the head and trunk of the chick embryo. Evolutionary implications

Our analysis of *Slug* overexpression all along the anteroposterior axis of the chick neural tube indicates that *Slug* is able to augment both the premigratory and migratory populations in the head. It should be stated here that the expression of several markers indicates that different cell populations were observed in the head region. Firstly, not all *Slug*- or *rhoB*-expressing cells were HNK-1 positive. As mentioned earlier, this may be the result of HNK-1 expression only being acquired in migratory crest cells at a distance from the neural tube. This explains the population of HNK-1 negative cells located close to the neural tube. However, HNK-1 is not expressed in some *Slug*- and/or *rhoB*-expressing neural crest cells located at different positions along the migratory path, suggesting that HNK-1 is not expressed in all migratory crest cells. Nevertheless, there is a significant population that only express HNK-1, in agreement with the fact that both *Slug* and *rhoB* are only transiently expressed in the migratory crest. The existence of these different populations has been confirmed in control embryos. This indicates that the excess of neural crest cells induced by *Slug* overexpression follow the normal migratory pathways and express the appropriate markers according to their location. This is in contrast to the phenotypes obtained after overexpressing Foxd3, since in this case, ectopic HNK-1 is observed at abnormal positions, including different dorsoventral levels of the neural tube (Kos et al., 2001).

The capacity of Slug to increase the migratory population is observed from the anterior head region to its border with the spinal cord. It is interesting to note here that we have observed an increase in cell migration up to the region of the fourth-fifth somite, exactly the axial level that has been mapped by chick-quail chimeras studies to correspond to the border between the hindbrain and the spinal cord (Cambronero and Puelles, 2000). Within the spinal cord, we have observed an extended area expressing the premigratory marker *rhoB* but we have not detected changes in the amount or the position of migratory cells. This indicates that different mechanisms may control the delamination of the neural crest in the head and in the trunk regions.

The demonstration that different mechanisms are involved in neural crest delamination in the head and the trunk have independent support from data being generated along the years in which clear differences were observed in these two regions with respect to the molecules used by the neural crest cells to attach to the extracellular matrix (Bronner-Fraser and Lallier, 1988; Lallier et al., 1992). In addition, the hyaluronate receptor CD44 is restricted to the neural crest of the cranial region (Corbel et al., 2000). Furthermore, the gradient of noggin expression that, complementary to that of BMP along the AP axis, has been proposed to control the time of trunk crest delamination (Sela-Donenfeld and Kalcheim, 1999) is not observed in the head. Since there is evidence of BMP signalling also being involved in crest delamination in the head (Kanzler et al., 2000), it is plausible that a different BMP signalling inhibitor may be implicated (Coffinier et al., 2001). Another very pertinent example of differences observed between head and trunk is that of *Pax*3. Whereas Slug is the first to be expressed in the head, Pax3 is expressed before Slug in the trunk region (Buxton et al., 1997a). This is compatible with our finding that *Slug* can induce *Pax3* expression in the hindbrain and it is incapable of doing so in the trunk region.

At stages at which *Slug* is already expressed in the neural folds, blocking of BMP signalling by Noggin inhibited neural crest delamination that was preceded by the downregulation of rhoB and cadherin 6B but had no effect on Slug expression (Sela-Donenfeld and Kalcheim, 1999). Thus, whilst BMP signalling is not needed to maintain *Slug* expression at these stages, Sela-Donenfeld and Kalcheim suggested that either *rhoB* and/or *cadherin 6B* expression are independent of Slug or that Slug was not sufficient to induce their expression. We have shown here that *rhoB* is directly or indirectly regulated by *Slug* and that *cadherin 6B* does not seem to be a target of Slug. However, independently of the induction of target gene expression and of the expanded area of crest produced within the neural tube, it is clear that *Slug* overexpression does not increase the delamination of neural crest in the trunk region.

Thus, it appears that Slug is involved in the specification of the neural crest precursors but not in crest emigration in the trunk region, as already suggested by Sela-Donenfeld and Kalcheim (Sela-Donenfeld and Kalcheim, 1999). This might have important evolutionary implications. The single *Snail* gene present in ascidian and amphioxus embryos is expressed at the edges of the neural plate and interestingly, in *Ciona, Snail* is also expressed in pigmented cells (Corbo et al., 1997;

Langeland et al., 1998; Wada and Saiga, 1999). Furthermore, the *Pax* gene representative of *Pax3* and *Pax7* both in amphioxus and ascidians, *AmphiPax3/7* and *HrPax37*, are also expressed in bilateral stripes along the edges of the neural plate (Holland et al., 1999, Wada et al., 1997). The expression of these neural crest markers in amphioxus and urochordates in the site of neural crest induction in vertebrates has led to the suggestion that they reveal a precursor population of the neural crest that is already specified in invertebrate chordates (Holland et al., 1999). In *Drosophila*, *snail* has been implicated in mesoderm specification (Boulay et al., 1987) and the non-vertebrate chordates also express *snail* in the early mesoderm. Thus, it is tempting to speculate that Snail genes played an ancestral role in the specification of tissues such as the mesoderm and the neural crest. The absence of Slug function in trunk crest emigration could derive from the acquisition of this function only in the cranial region. Taken together, our results indicate that the involvement of the *Snail* gene family in neural crest development is conserved throughout evolution.

From their specification to their delamination, mediated by the triggering of EMT, the formation of the neural crest is a developmental process intrinsically linked to the Snail family. The EMT necessary for delamination may be carried out by *Slug* or *Snail* depending on the vertebrate species (see below). In addition, they have been co-opted for the induction of EMTs needed for other morphogenetic processes such as the formation of heart cushions (Romano and Runyan, 2000) or pathological situations such as the malignisation of epithelial tumours (Cano et al., 2000; Batlle et al., 2000). Since the appearance of the neural crest together with that of the placodes are believed to be fundamental in the emergence of the vertebrate head (Gans and Northcutt, 1983), the Snail gene family is situated in a privileged position with regard to the study of morphogenesis in vertebrates.

Finally, it is worth considering that cranial neural folds contain a distinct population of cells with different fate than cells derived from the dorsal neural tube in the trunk. Since only cranial neural crest contribute to cartilage and bone, the possibility that these cells have a unique responsiveness to Slug function cannot be discarded.

Functional equivalence of Snail family members in neural crest development

The induction of EMT is the process that first identified *Slug* as being critical for neural crest and mesoderm delamination in the chick embryo (Nieto et al., 1994). The normal mesoderm and neural crest development in a *Slug* null mutant mouse compromised the importance of the role of Slug in these processes (Jiang et al., 1998). However, *Slug* is not expressed in the premigratory neural crest or in the primitive streak of the mouse, while the other family member, *Snail*, is found at these sites. This very striking interchange in the expression patterns of the two family members (*Slug* and *Snail*) between chicken and mouse embryos led to the suggestion that *Snail* rather than *Slug* could be the gene involved in EMT in the mouse (Sefton et al., 1998). The confirmation that this is the case, has come from the ability of mouse *Snail* to trigger a complete EMT in epithelial cell lines (Cano et al., 2000; Batlle et al., 2000) and the fact that *Snail* knockout embryos die at gastrulation as a result of defects in EMT during mesoderm formation (Carver et al., 2001).

It is interesting to note that the interchange in the expression patterns is not complete, but occurs in the sites of EMT throughout the developing embryo (Sefton et al., 1998) (unpublished observations). This raises the question of how this unusual phenomenon occurred in evolution. But firstly, the question that has to be addressed is whether the two of them are functionally equivalent when ectopically expressed at the appropriate sites both intra- and interspecies. Chick *Snail* was capable of mimicking the effect of *Slug* overexpression when ectopically expressed in the chick hindbrain, a region where it is not expressed at these stages (Sefton et al., 1998) (unpublished observations). However, the effect of this ectopic expression was milder than that observed by overexpressing the endogenous gene. This indicates that *Snail* and *Slug* can be functionally equivalent in the development of the neural crest. Similar results were obtained after ectopically expressing mouse *Snail*, demonstrating that this functional equivalence is also maintained within different vertebrate species. These transcription factors are composed of two main domains, a DNA binding domain essentially identical among all *Snail* and *Slug* vertebrate family members and a much more divergent putative protein-protein interaction domain. Hence, the challenge that remains is to determine the mechanisms used by these two proteins to fulfil the same role during evolution with respect to their target genes, their regulatory partners and the genetic cascades in which they are involved.

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