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Neural crest ontogeny during secondary neurulation: a gene expression pattern study in the chick embryo

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ABSTRACT In the prospective lumbo-sacral region of the chick embryo, neurulation is achieved by cavitation of the medullary cord, a process called secondary neurulation. Neural crest cells (NCC) are generated in this region and they give rise to the same types of derivatives as in more rostral parts of the trunk where neurulation occurs by dorsal fusion of the neural plate borders (primary neurulation). However, no molecular data were available concerning the different steps of their ontogeny. We thus performed a detailed expression study of molecular players likely to participate in the generation of secondary NCC in chick embryos between Hamburger and Hamilton stages 18-20 (HH18-20) at the level of somites 30 to 43. We found that specification of secondary NCC involves, as in primary neurulation, the activity of several transcription factors such as *Pax3*, *Pax7*, *Snail2*, *FoxD3* and *Sox9*, which are all expressed in the dorsal secondary neural tube as soon as full cavitation is achieved. Moreover, once specification has occurred, emigration of NCC from the dorsal neuroepithelium starts facing early dissociating somites and involves a series of changes in cell shape and adhesion, as well as interactions with the extracellular matrix. Furthermore, *Bmp4* and *Wnt1* expression precedes the detection of migratory secondary NCC and is coincident with maturation of adjacent somites. Altogether, this first study of molecular aspects of secondary NCC ontogeny has revealed that the mechanisms of neural crest generation occurring along the trunk region of the chick embryo are generally conserved and independent of the type of neurulation involved.

KEY WORDS: *epithelium-to-mesenchyme transition, delamination, BMP/WNT signaling*

Neurulation, the developmental process leading to the formation of the vertebrate neural tube (NT), classically follows two spatially and temporally distinct processes in amniote embryos. In the anterior part of the body, which corresponds to the cephalic and cervico-thoracic regions, neurulation involves bending of the neural plate, a thickened epithelium that rapidly folds up into a groove, giving rise to a tube by dorsal fusion of its lateral borders. This process, called primary neurulation, ends up as early as 15-somite stage (stage 12 of Hamburger and Hamilton (1951) (HH12)) at the prospective level of the 27th pair of somites, where the posterior neuropore is located in the chick embryo (Schoenwolf, 1979). In contrast, during secondary neurulation, occurring at the level of the presumptive lumbo-sacro-caudal region, formation of the NT occurs by cavitation of a solid rod of cells, the medullary cord (Schoenwolf and Delongo, 1980; Catala *et al.*, 1995). Fate mapping and transplantation experiments have shown that, despite the morphological differences which distinguish these two modes of neurulation, the same mechanisms are involved as far as the elongation process of the neural tube is concerned (Catala *et al.*, 1995; Catala *et al.*, 1996; Wilson and Beddington, 1996; Charrier *et al.*, 2005).

We are here focusing on the neural crest (NC), a transient structure appearing in the dorsal NT shortly after its formation, and giving rise to a population of migrating cells that generate a great set of distinct structures according to their origin along the antero-posterior (AP) axis. In the avian embryo, NC cells (NCC)

Abbreviations used in this paper: AP, antero-posterior; DSHB, Developmental Study Hybridoma Bank; E, embryonic day; ECM, extracellular matrix; EDS, early dissociating somites; EMT, epithelium-to-mesenchyme transition; HH, Hamburger and Hamilton; NC, neural crest; NCC, neural crest cells; NT, neural tube; PSM, presomitic mesoderm.

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arising from the cephalic levels give rise to mesectodermal derivatives (such as skeleton of the face and skull, dermis of the head and neck, connective tissue and tendons of the face and eye muscles, and meninges of the forebrain). They also produce neural and glial derivatives that yield the cephalic peripheral (sensory and parasympathetic) nervous system, and melanocytes. In contrast, at the trunk level, avian NCC do not give rise to mesectodermal derivatives but generate Schwann cells and neurons of the peripheral nervous system (sensory, sympathetic and parasympathetic), medullary cells of the adrenal glands and melanocytes (see Le Douarin and Kalcheim, 1999 for a review). In primary neurulation, the NC primordium corresponds to the border between neural and non-neural ectoderm and then, as neural folds fuse, it becomes located in the dorsal aspect of the NT. Once specified by a distinct program of gene expression, these cells undergo an epithelium-to-mesenchyme transition (EMT), delaminate from the neuroepithelium and migrate into the periphery where they differentiate (see Kalcheim and Burstyn-Cohen, 2005 for a review). As a consequence of its proper mode of formation, the dorsal region of the secondary NT is not issued from the fusion of neural folds, but it also gives rise to NCC contributing to the same types of cells as its more rostral trunk counterpart (Schoenwolf *et al.*, 1985; Catala *et al.*, 1995; Catala *et al.*, 2000).

During the last decades, great progresses have been made in the identification of the molecules and mechanisms involved in the different steps leading to the formation of the primary NC. However, generation of NCC from the secondary NT has been the subject of much less analysis and no molecular data are available concerning the several aspects of the formation of these cells. This prompted us to perform, at the level of the secondary neurulation, a detailed study of expression of a large set of molecules known to act in the different steps of NC genesis. Our study was carried out in chick embryos from HH18 to HH20, during the 3rd day of embryonic development (E3). From these stages onwards, morphogenesis and growth of the caudal part of the body result from the development of the tail bud, the region located caudally to the posterior neuropore (Schoenwolf, 1979; Catala *et al.*, 1995). We have focused our analysis in the most recently orga-

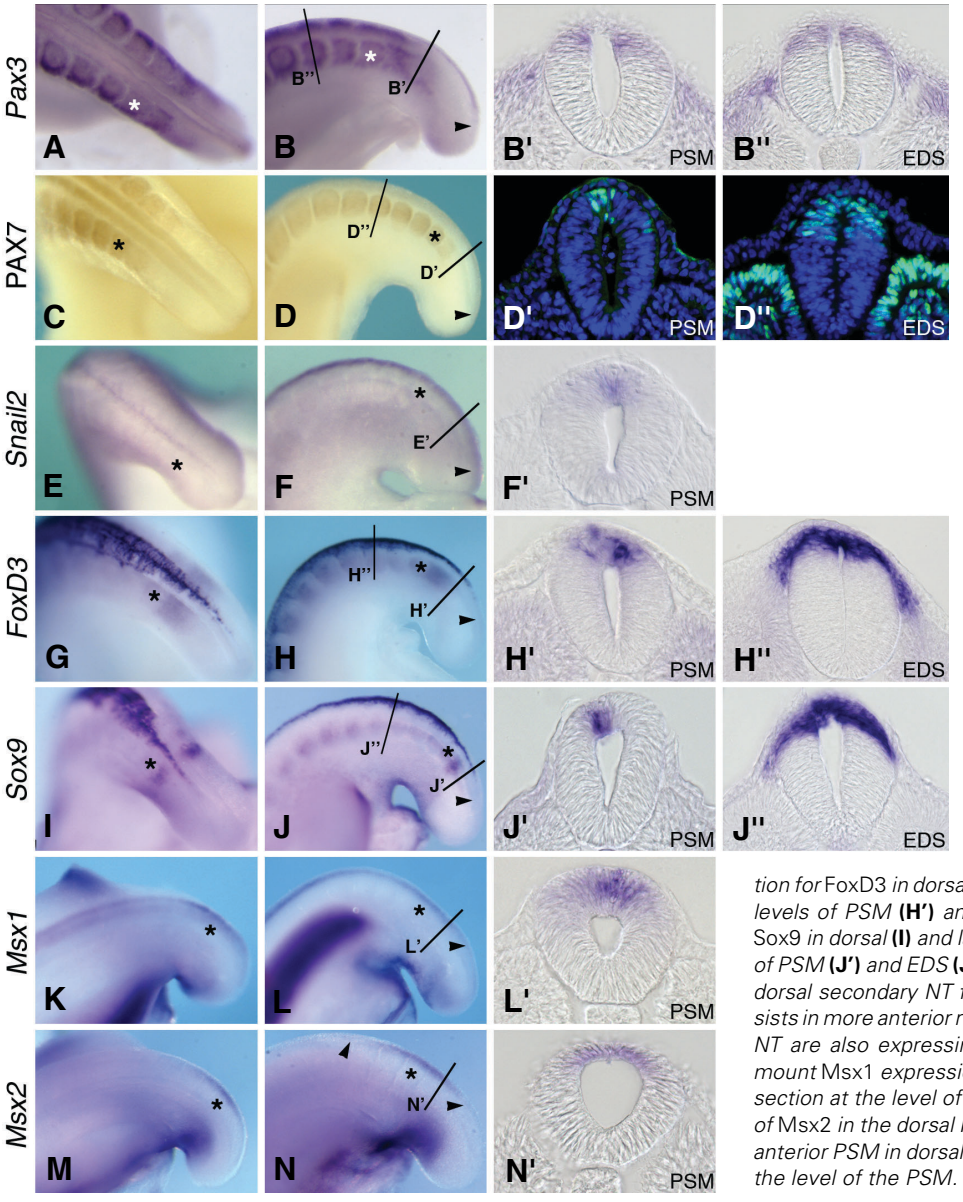

Fig. 1. Specification of secondary neural crest cells in HH18-20 chick embryos. (A,B) Pax3 *expression in whole mount, dorsal view* **(A)** *and lateral view* **(B)**. *Asterisk indicates the last formed somites and arrowhead points the posterior limit of expression. Cross-sections at the level of presomitic mesoderm (PSM)* **(B')** *and early dissociating somites (EDS)* **(B'')**. **(C,D)** *PAX7 immuno-detection in whole mount dorsal* **(C)** *and lateral* **(D)** *and cryo-sections at the levels of PSM* **(D')** *and EDS* **(D'')**. *Note that both transcription factors are detected in the dorsal NT soon after its formation facing posterior PSM and persist on migrating NCC close to the dorsal NT. They are also detected in the somite cells* ***(B'',D'')***. ***(E,F)*** *Whole mount expression of* Snail2 *in dorsal view* **(E)** *and lateral view* **(F)**. Snail2 *is expressed in the dorsal NT from the level of the 9th-10th last formed somites down to the posterior PSM seen in cross-section* **(F')**. **(G,H)** *Whole mount* in situ *hybridization for* FoxD3 *in dorsal* **(G)** *and lateral* **(H)** *views. Cross-sections at the levels of PSM* **(H')** *and EDS* **(H'')**. **(I,J)** *Whole mount expression of* Sox9 *in dorsal* **(I)** *and lateral* **(J)** *views and cross-sections at the levels of PSM* **(J')** *and EDS* **(J'')**. FoxD3 *and* Sox9 *are firstly expressed in the dorsal secondary NT facing posterior PSM and their expression persists in more anterior regions. Early migrating NCC located close to the NT are also expressing both of these genes* **(H'',J'')**. **(K,L)** *Whole mount* Msx1 *expression in dorsal* **(K)** *and lateral* **(L)** *views.* **(L')** *Cross-section at the level of anterior PSM.* **(M,N)** *Whole mount expression of* Msx2 *in the dorsal NT from the 4th last formed somite down to the anterior PSM in dorsal* **(M)** *and lateral* **(N)** *views.* **(N')** *Cross-section at the level of the PSM.*

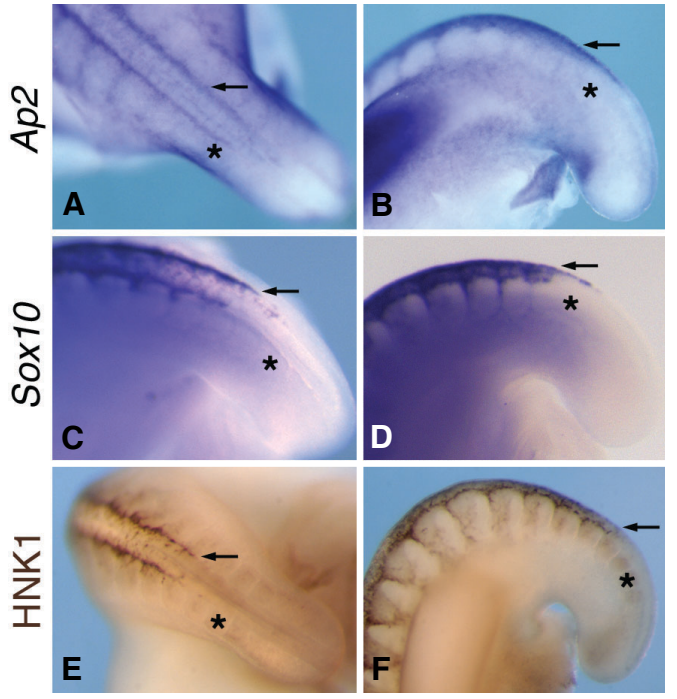

Fig. 2. Early migration of secondary neural crest cells in HH18-20 chick embryos. (A,B) *Whole mount* in situ *hybridization for* Ap2 *in dorsal* **(A)** *and lateral* **(B)** *views showing NCC emigrating at the level of the 2nd last formed somite (arrows).* **(C,D)** *Whole mount* in situ *hybridization for* Sox10 *in dorsal (C) and lateral (D) views, with migrating NCC detected at the level of the 3rd last formed somite (arrows)* **(E,F)** *Immuno-detection in whole mount for HNK1 in dorsal* **(E)** *and lateral* **(F)** *views.*

nized region of these embryos corresponding to the level of future somites 30 to 43, namely the non-segmented area, the last formed epithelial somites and early dissociating somites (EDS), in which one can follow the successive stages of NC maturation along a caudo-rostral direction.

We have analyzed the expression pattern of a great number of transcription factors implicated in the specification of the NC (see Sauka-Spengler and Bronner-Fraser, 2006 for a review). All of them are detected in the dorsal secondary NT a short time after this one is fully formed and they exhibit a differential expression according to the NT state of maturation along its caudo-rostral axis. Genes like *Pax3* and *Pax7* (Fig. 1A-D) are detected dorsally in the recently formed NT, soon after cavitation is completed, in a level corresponding to posterior presomitic mesoderm (PSM) (Fig. 1B',D'). More rostrally, their expression continues in the dorsal region of the NT and they are also transiently expressed by NCC that have recently emigrated from the neuroepithelium (Fig. 1B",D"). *Snail2* (previously known as *Slug*) is expressed transiently in the secondary NT, soon after its full cavitation, from the level of posterior PSM (Fig. 1E-F') up to the 9th-10th last formed somites (not shown). We found that *FoxD3* and *Sox9* (Fig. 1G-J), considered as the "NC-specifying" genes together with *Snail2* (Cheung *et al.*, 2005), are also expressed dorsally in the recently formed secondary NT. However, their first detection in the posterior PSM level is slightly more rostral (Fig. 1H',J') than that of *Pax3*, *Pax7* and *Snail2*. Contrarily to this last one, which ends at about the 10th last formed somite, their expression persists more rostrally (not shown). Moreover, they are transiently present in the early migrating NCC located close to the NT (Fig. 1H",J"). In contrast, *Msx1* and *Msx2* are firstly detected in the dorsal NT located at the level of the anterior PSM (Fig. 1K-N'). Besides, we have found that *Msx1* persists in the more rostral NT (Fig. 1L), while *Msx2* is only detected transiently, disappearing at the level of the 4th last formed somite (Fig. 1N).

Once they are specified, emigrating NCC acquire specific markers. The first of them, *Ap2*, is detected in the dorsal NT at the level of the anterior PSM like the markers of NC specification (Fig. 2A-B) and it persists in the NCC that have recently emigrated from the neuroepithelium at the level of the 2nd last formed somite (Fig. 2 A,B). Other markers, such as *Sox10* and HNK1, label migratory secondary NCC somewhat later. While *Sox10* appears at the level of the 3rd last formed somite (Fig. 2C,D), HNK1 is apparent at the transition between the 3rd and 4th last formed somite (Fig. 2E,F). In sum, although *Ap2* and other markers of early migration (such as *Pax3*, *Pax7*, *FoxD3* and *Sox9*) show secondary NCC emigrating in front of the 2nd last formed somite, other markers like HNK1 give results similar to that reported in primary neurulation regions: in 15 to 25-somite stage (HH11 to HH15) chick embryos, onset of NCC migration occurs at the level of the 3rd-4th last formed somites (Tosney, 1978; Thiery *et al.*, 1982; Teillet *et al.*, 1987).

We have then examined the delamination of secondary NCC, a process that involves, in the cervico-thoracic region, a series of cellular events that are similar to those occurring in any EMT (see Duband, 2006 for a review). We have observed that *RhoB*, a small GTPase proposed to control cell adhesion and motility (Liu and Jessel, 1998), is detected along the entire length of the secondary NT (Fig. 3B), in a dorso-ventral gradient (Fig. 3A), which is visible

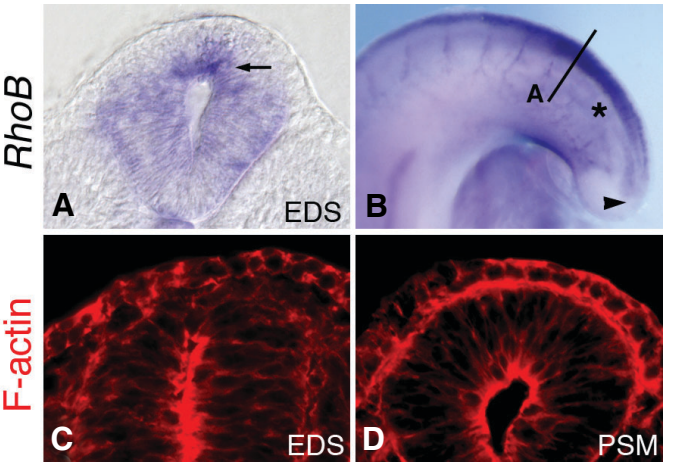

Fig. 3. *RhoB* expression and cell shape changes during epithelium-to-mesenchyme transition of secondary neural crest cells in HH18-20 chick embryos. (A,B) In situ *hybridization for* RhoB *in cross-section at the level of early dissociating somites (EDS) and whole mount. This small GTPase is expressed in a dorso-ventral gradient* **(A)** *along the entire length of secondary neural tube (NT)* **(B)**. *Note the polarized distribution of the transcripts in the apical side of the dorsal neuroepithelial cells (arrow). Asterisk points to the last somite formed and arrowhead points the posterior limit of expression.* **(C)** *During emigration of NCC at the level of EDS, phalloidin immunolabeling shows a loss of accumulation of F-actin in the apical side of the cells of the dorsal NT.* **(D)** *Before onset of NCC emigration at the level of presomitic mesoderm (PSM), these cells exhibit the typical apico-basal polarity of neuroepithelial cells.*

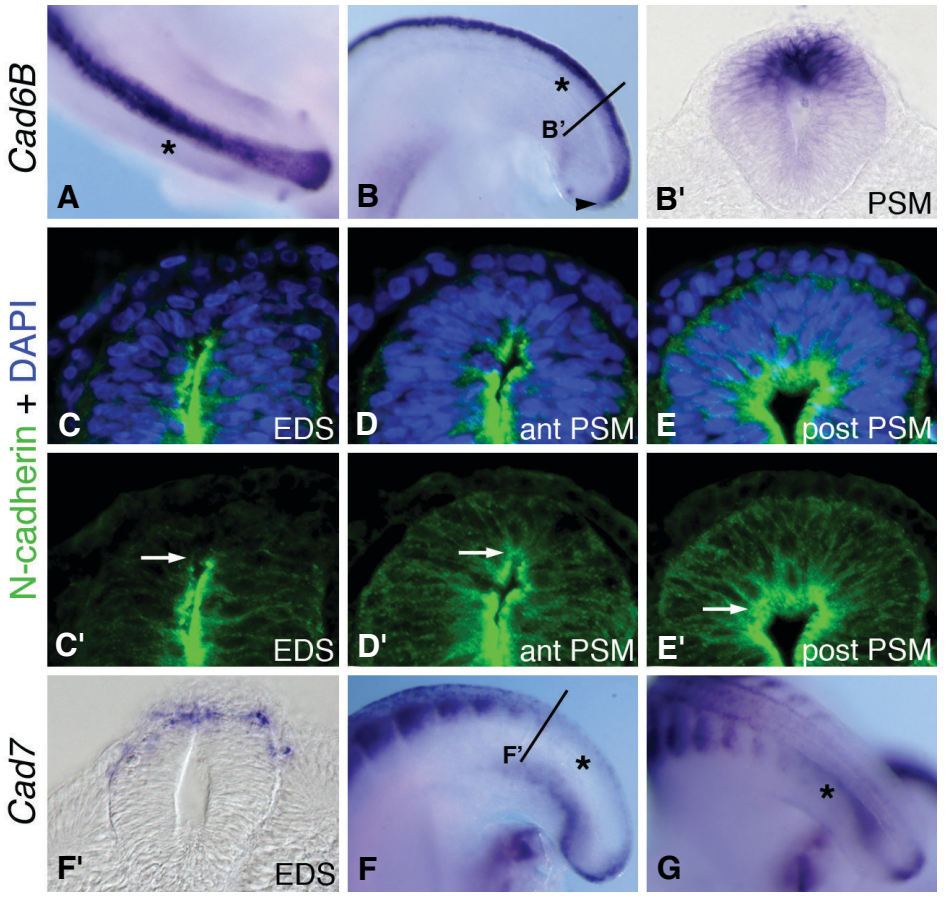

Fig. 4. Differential cadherin expression during epithelium-to-mesenchyme transition of secondary neural crest cells in HH18-20 chick embryos. (A,B) *Expression of* Cad6B *all along the secondary neural tube (NT) down to the tail bud detected by whole mount* in situ *hybridization in dorsal* **(A)** *and lateral* **(B)** *views.* **(B')** *Cross-section at the level of the presomitic mesoderm (PSM) showing that this gene is expressed in the dorsal region of the NT. Asterisk represents the last formed somite and arrowhead points the posterior limit of expression.* **(C-E')** *Immuno-detection of N-cadherin on cryo-sections, counterstained with DAPI* **(C-E)**. *The accumulation of N-cadherin in the dorsal cells of the secondary NT at the posterior PSM level* **(E,E')** *is progressively decreased in the anterior PSM* **(D,D')** *and in early dissociating somite (EDS)* **(C,C')** *levels. Arrows point the decreased apical accumulation of N-cadherin on dorsal cells of the secondary NT as this one maturates* **(C'-E')**. **(F'-G)** *Once migrating, secondary NCC express* Cad7*, detected by* in situ *hybridization in cross-section at the level of EDS* **(F')** *and in whole mount lateral* **(F)** *and dorsal* **(G)** *views.*

soon after NT formation at a level facing the posterior PSM (not shown). Moreover, we have evidenced that *RhoB* expression is polarized in the dorsal cells of the NT facing EDS, where transcripts are accumulated in their apical side (Fig. 3A). Emigrating secondary NCC do not express *RhoB* (not shown). This pattern of expression of *RhoB* is quite different from that described in more anterior regions at equivalent stage. For example, it has been shown that, in 10-somite stage (HH10) chick embryos, this molecule is initially expressed in the dorsal tips of the neural folds, dorsal NT and transiently in migrating NCC located close to the NT (Liu and Jessel, 1998). To determine if the differences that we have ascertained are due to different staining methodology, we have analyzed *RhoB* expression during primary neurulation in 17- to 18-somite stage embryos (HH12-13) and we have obtained results similar to that of Liu and Jessel (1998) (not shown). Thus, *RhoB* is differently expressed during primary and secondary neurulation. Since it is assumed that this molecule participates in EMT of NCC through dynamic regulation of actin cytoskeleton (Liu and Jessel, 1998), we decided to analyze the distribution of F-actin in the context of secondary neurulation. We have found that F-actin is accumulated both in the basal and in the apical sides of the dorsal cells of the secondary NT before onset of migration of the NCC at the level of PSM (Fig. 3D). Once migration starts at the level of EDS, the basal accumulation of F-actin is lost whereas the apical one continues to be observed (Fig. 3C). Thus, the reorganization of the actin cytoskeleton, contributing to the cell shape changes that precede emigration of NCC (Newgreen and Minichiello, 1996), is similar in secondary and primary neurulation regions, although the distinct pattern of expression of *RhoB*, proposed to control actin cytoskeleton (Liu and Jessell, 1998), in these two regions.

We have also observed signs of significant changes in the cellular adhesion of dorsal cells of secondary NT. Long before NCC emigration, the dorsal cells of the secondary NT express *Cad6B* (Fig. 4A,B'). Moreover, N-cadherin, present in the dorsal cells of the secondary NT located at the level of the posterior PSM (Fig. 4E,E'), progressively disappears from these cells, before and during NCC emigration as seen respectively at anterior PSM (Fig. 4D,D') and EDS (Fig. 4C,C') levels. Once migrating, secondary NCC, first seen at the level of 3rd last formed somite, express *Cad7* (Fig. 4F-G').

We have ascertained the status of several extracellular matrix (ECM) components during generation of secondary NCC. As shown by laminin immuno-detection, the basement membrane of the secondary NT is partially absent (dotted interruptions) in the dorsal portion of the secondary NT since its formation at the level of the posterior PSM (Fig. 5B). This lack of a complete basement membrane persists during emigration of NCC (Fig. 5A). In contrast, concomitantly with the dorsal interruption of the basal lamina, we have observed that two main components of the fibrillar ECM, fibronectin and tenascinC, are present all around the secondary NT and along the migratory pathways of the NCC before (Fig. 5D,F) and during (Fig.

5C,E) emigration of these cells. Coincident with the changes in both the cell shape (see Figure 3) and adhesion (see figure 4), we have observed that the distribution of TASC, the activated form of beta1-integrin, is also modified as emigration of secondary NCC occurs. At the level of the PSM, we have found that TASC has a polarized distribution in the basal side of the dorsal cells of the secondary NT (Fig. 5H). Loss of this basal accumulation is observed as emigration of NCC starts at the level of EDS (Fig. 5G). Altogether, our observations show that delamination of secondary NCC involves the same cellular events that those occurring during emigration of NCC at the level of primary neurulation.

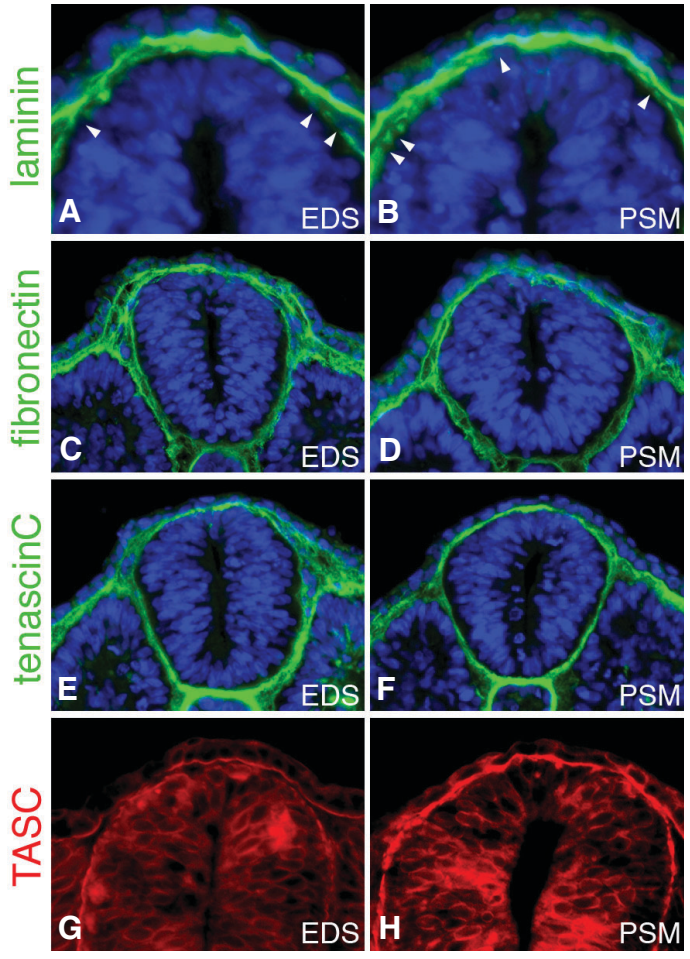

Fig. 5. Properties of the extracellular matrix during epithelium-to-mesenchyme transition of secondary neural crest cells in HH18-20 chick embryos. (A,B) *Laminin immuno-detection on cryo-setions at the levels of presomitic mesoderm (PSM)* **(B)** *and early dissociating somites (EDS)* **(A)**. *Basement membrane of the dorsal secondary neural tube (NT) is not complete both before* **(B)** *and during* **(A)** *emigration of neural crest cells (NCC) (arrowheads).* **(C-F)** *Immuno-fluorescence for fibronectin* **(C,D)** *and tenascinC* **(E,F)** *on cryo-sections, showing that both of these molecules are present in the extracellular matrix surrounding the secondary NT and along the migration pathways of NCC before* **(D,F)** *and during* **(C,E)** *emigration of these cells.* **(G,H)** *TASC, the activated form of beta1-integrin detected by immuno-histochemistry on cryo-sections, presents a polarized distribution in the apical side of the cells of the dorsal secondary early NT* **(H)** *that is lost as migration of NCC occurs* **(G)**.

Additionally to an EMT, onset of trunk NCC migration normally requires a BMP-dependent of WNT activity (Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000; Burstyn-Cohen *et al.*, 2004; Shoval *et al.*, 2007). We have thus continued our study about the molecular aspects of generation of secondary NCC by analysis of such pathways. *Bmp4* is expressed in the secondary dorsal NT along a rostro-caudal gradient that vanishes at the level of the posterior PSM (Fig. 6A-B''). Moreover, one of its inhibitors, *Noggin*, has a complementary pattern of expression (Fig. 6C-D"), being detected in the recently formed dorsal NT, from the level of the posterior PSM (Fig. 6D") up to the 1st-2nd last formed somites (Fig. 6C-D). *Noggin* is no longer detected as somites dissociate (Fig. 6D') and this is coincident with the detection of migratory NCC (see figure 2). Furthermore, we observed that expression of *Wnt1* in the dorsal secondary NT starts at the level of the 1st-2nd last formed somite (Fig. 6F') where *Noggin* is no longer detected (Fig. 6D'). *Wnt1* expression persists in more anterior regions (Fig. 6E,F). As in primary neurulation (Burstyn-Cohen *et al.*, 2004), down-regulation of *Noggin*, coincident with the initiation of *Wnt1* expression, slightly precedes emigration of secondary NCC.

In the present study, we have described the different steps involved in the generation of the secondary NCC in the lumbo-sacral region, at the level of somites 30 to 43, in the chick embryo. Several transcription factors that have been implicated in the specification of the trunk NCC are expressed in the dorsal region of the secondary NT shortly after its formation (full cavitation). Genes like *Pax3*, *Pax7* and *Snail2* are detected early at the level of the posterior PSM, while others (such as *Msx1* and *Msx2*) are detected later at the anterior PSM level. Moreover, once specification of secondary NCC has occurred, emigration of these cells from the neuroepithelium starts facing EDS. Secondary NCC delamination involves a series of cellular events, such as reorganization of the F-actin cytoskeleton that contributes to changes in the shape of the premigratory cells. Furthermore, a dynamic regulation of the adhesion of secondary NCC was also observed, as shown by the differential expression of *Cad6B*, N-cadherin and *Cad7* by these cells. All these changes are accompanied by a modification of interaction with the components of the ECM, as demonstrated by the distribution of TASC in the dorsal cells of the secondary NT. In addition, we have shown that the acquisition of motility by secondary NCC is coincident with an increased activity of *Bmp4*, through both an increase in the proper transcripts as well as down-regulation of its inhibitor *Noggin*, and *de novo* expression of *Wnt1*. These events are related with the maturation of the adjacent somites, since migratory NCC are firstly detected at the level of early dissociating somites. Altogether, our work points out for the first time the molecular aspects of the different steps involved in the formation of secondary NCC. In addition, a careful comparison of the onset of expression of the several genes analysed allows us to propose a molecular hierarchy operating during ontogeny of secondary NC (Fig. 7). The analysis of the great set of molecules that we have performed suggests that the molecular code and mechanisms acting in the different stages of NC generation are similar along the trunk axis in the chick embryo, independently of the type of neurulation that leads to the formation of the NT. However, our results

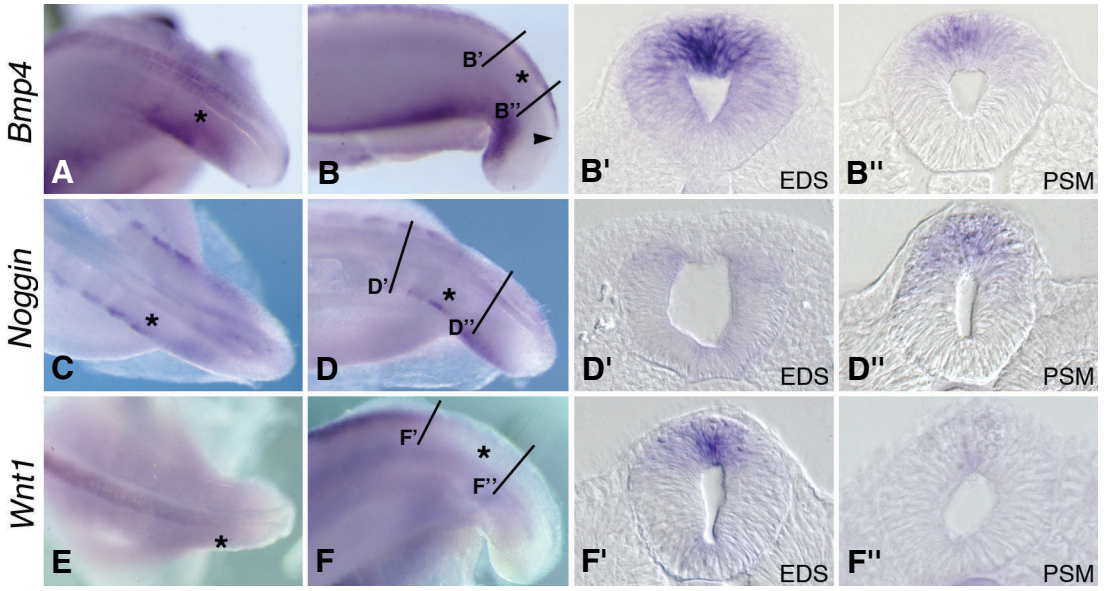

Fig. 6. Secondary neural crest cell migration follows BMP and WNT signaling in HH18-20 chick embryos. In situ *hybridization of* Bmp4 **(A,B)**, Noggin **(C,D)** *and* Wnt1 **(E,F)** *in whole mount embryos in dorsal* **(A,C,E)** *and lateral* **(B,D,F)** *views, and cross-sections at the levels of the early dissociating somites (EDS)* **(B',D',F')** *and presomitic mesoderm (PSM)* **(B'',D'',F'')**. *Asterisks indicate the last formed somite and arrowhead points the posterior limit of expression. Note that* Bmp4 *and* Noggin *are expressed in a complementary rostro-caudal gradient along the dorsal secondary neural tube (NT).* Bmp4 *is detected down to the level of posterior PSM,* **(A-B'')**, *while* Noggin *is detected facing posterior PSM and down-regulated at the level of the 1st-2nd last formed somites* **(C-D'')**. *Increase in* Bmp4 *activity is accompanied by the expression of* Wnt1 *that persists in the dorsal NT in more anterior regions* **(E-F'')**.

also put forward several differences relative to generation of NC in the head. These include variations in the onset of expression of genes such as *Pax3*, *Snail2* and *RhoB*. In the cephalic region, *Snail2* precedes the expression of *Pax3* (del Barrio and Nieto, 2002) and *RhoB* is present in a small population of *Snail2*-expressing cells (del Barrio and Nieto, 2004). We have found that during secondary neurulation, onset of *Pax3* and *Snail2* expression in the dorsal aspect of the secondary NT are concomitant, with *RhoB* being detected earlier than these two transcription factors. Furthermore, *Cad6B* is quickly down-regulated in the head by *Snail2* (del Barrio and Nieto, 2002; Coles *et al.*, 2007) while it is maintained in the secondary NT after onset of NCC emigration (this study). Moreover, the mechanisms triggering NCC delamination are also different in the head and trunk regions. In both primary and secondary trunk NT, emigration of NCC from the neuroepithelium involves a *Bmp4* activity regulated by a gradient of *Noggin* expression in the dorsal NT (Sela-Donenfeld and Kalcheim, 1999; this study). In contrast, typical delamination of NCC in the head region involves the activity of *Snail2* and *Ets1* (Theveneau *et al.*, 2007) and not that of *Bmp4*. Thus, our results about formation of secondary NCC emphasize some differences in the development of NC in the cephalic *versus* trunk region. However, along the trunk, despite the obvious morphological differences between primary and secondary neu-

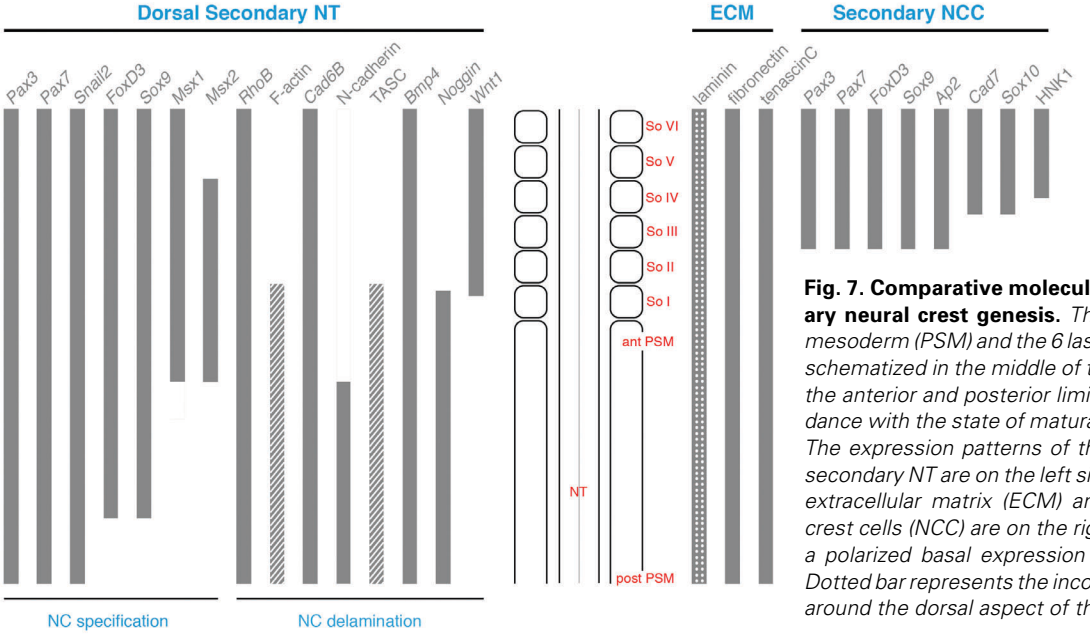

Fig. 7. Comparative molecular expression during secondary neural crest genesis. *The neural tube (NT), presomitic mesoderm (PSM) and the 6 last formed somites (So I to VI) are schematized in the middle of the figure. Vertical bars indicate the anterior and posterior limits of gene expression in accordance with the state of maturation of the paraxial mesoderm. The expression patterns of the genes present in the dorsal secondary NT are on the left side, while those occurring in the extracellular matrix (ECM) and migratory secondary neural crest cells (NCC) are on the right side. Striped bars represent a polarized basal expression in the dorsal cells of the NT. Dotted bar represents the incomplete accumulation of laminin around the dorsal aspect of the secondary NT.*

rulation, identical mechanisms are implicated in NC formation.

Materials and Methods

Chick and quail embryos

Fertilized chick (*Gallus gallus domesticus*) eggs of commercial sources were incubated at 38°C in humidified atmosphere. Embryos were staged according to Hamburger and Hamilton (HH) table (1951) and/or referred to embryonic day (E). Control and experimental embryos were fixed in 4% paraformaldehyde either 2 hours at room temperature (RT) for immunohistochemistry, or overnight (ON) at 4°C for *in situ* hybridization. For tissue sections, fixed embryos were cryo-protected in 15% sucrose ON at 4°C, embedded in gelatin, frozen and conserved at -20°C. Serial 8, 12 or 30 μm sections were performed in a Leica cryostat and mounted on glass slides.

In situ *hybridization in whole embryos and sections*

Whole mount *in situ* hybridizations were performed according to Henrique and collaborators (1995) with the following chick-specific riboprobes: *Ap2* (Shen *et al.*, 1997), *Bmp4* (Francis-West *et al.*, 1994), *Cad6B* and *Cad7* (Nakagawa and Takeichi, 1995), *FoxD3* (Dottori *et al.*, 2001; Kos *et al.*, 2001), *Msx1* (Coelho *et al.*, 1992) and *Msx2* (Coelho *et al.*, 1991), *Noggin* (Reshef *et al.*, 1998), *Pax3* (Goulding *et al.*, 1993), *RhoB* (Liu and Jessel, 1998), *Snail2* (Nieto *et al.*, 1994), *Sox9* (Cheung and Briscoe, 2003), *Sox10* (Cheng *et al.*, 2000) and *Wnt1* (Megason and McMahon, 2002). Once photographed, embryos were processed for cryo-sectioning as previously described.

Immunohistochemistry in whole embryos and sections

Immunohistochemistry was performed as previously described (Afonso and Catala, 2005) using the following primary antibodies: anti-N-cadherin 1:500 (FA-5, Sigma), anti-fibronectin 1:200 (Rovasio *et al.*, 1983; kindly provided by Dr. Jean-Loup Duband), anti-activated beta1-integrin (TASC) 1:100 (MAB19294, Chemicon), anti-laminin 1:100 (L9393, Sigma), anti-NC1/HNK1 1:20 (Vincent *et al.*, 1983; Vincent and Thierry, 1984; Tucker *et al.*, 1984; kindly provided by Dr. Jean-Loup Duband), anti-PAX7 1:20 (Developmental Study Hybridoma Bank, (DSHB)), anti-tenascinC 1:300 (AB19013, Chemicon). For whole mount immunohistochemistry, species-specific secondary antibodies were conjugated with HRP (1:100, Southern Biotechnologies). Once photographed, embryos were processed for cryo-sectioning as previously described. For immunofluorescence in sections we used secondary antibodies conjugated with FITC or TRITC (1:200, Southern Biotechnologies). For F-actin labeling, we have used phalloidin conjugated with TRITC 1:1000 (P1951, Sigma). DAPI 1:5000 (D1306, Molecular Probes) for counterstaining.

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