

Delamination of cells from neurogenic placodes does not involve an epithelial-to-mesenchymal transition

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Neurogenic placodes are specialized regions of embryonic ectoderm that generate the majority of the neurons of the cranial sensory ganglia. Here we examine in chick the mechanism underlying the delamination of cells from the epibranchial placodal ectoderm. We show that the placodal epithelium has a distinctive morphology, reflecting a change in cell shape, and is associated with a breach in the underlying basal lamina. Placodal cell delamination is distinct from neural crest cell delamination. In particular, exit of neuroblasts from the epithelium is not associated with the expression of *Snail/Snail2* or of the Rho family GTPases required for the epithelial-to-mesenchymal transition seen in neural crest cell delamination. Indeed, cells leaving the placodes do not assume a mesenchymal morphology but migrate from the epithelium as neuronal cells. We further show that the placodal epithelium has a pseudostratified appearance. Examination of proliferation shows that the placodal epithelium is mitotically quiescent, with few phosphohistone H3-positive cells being identified. Where division does occur within the epithelium it is restricted to the apical surface. The neurogenic placodes thus represent specialized ectodermal niches that generate neuroblasts over a protracted period.

KEY WORDS: Placode, Delamination, Sensory neuron, Neural crest, EMT

INTRODUCTION

The neurogenic placodes generate the majority of neurons of the cranial sensory ganglia, including all of those mediating the special senses of hearing, balance and taste (D'Amico-Martel and Noden, 1983; Graham and Begbie, 2000). These placodes arise at stereotypical positions in the head of vertebrate embryos as the result of localized inductive signals (Begbie and Graham, 2001a). The neuronal precursors that are generated in each of the placodes express genes indicative of their later sensory phenotype, even though the cells delaminating from all of the placodes, except the ophthalmic trigeminal, are mitotically active (Begbie et al., 2002). The placodal neuroblasts are guided internally by neural crest streams to the sites of ganglion formation where they terminally differentiate (Begbie and Graham, 2001b). We have accumulated a body of knowledge about the induction of epibranchial placodes and about how the migration of the neuroblasts is organized, yet little is known about how these cells actually leave the placodal epithelium.

The best-studied example of delamination in the vertebrate embryo is the emergence of neural crest cells from the neural tube. This transient population of multipotent cells is born in the dorsal aspect of the neural tube, and then migrates into the periphery where they generate a wide range of derivatives including sensory neurons. To leave the epithelium, the crest cells alter their contact with the other cells in the dorsal neural tube and assume a mesenchymal morphology, i.e. they undergo an epithelial-to-mesenchymal transition (EMT) (Le Douarin, 1999). The emigration of these cells also involves a breach in the basal lamina around the dorsal aspect of the neural tube, which subsequently seals. The process of neural crest cell delamination has been shown to be transcriptionally controlled by the *Snail* family of transcription factors (Nieto, 2002),

and to require the small GTPase RhoB (Liu and Jessell, 1998). Importantly, there have been suggestions that the neural crest and the neurogenic placodes exhibit a number of similarities. Both tissues share an ectodermal origin and give rise to migratory cells, and the likelihood of common developmental mechanisms has been suggested (McCabe et al., 2004). We have therefore carried out a detailed analysis of cell delamination from the epibranchial placodes and compared this process with neural crest delamination.

Here we present data that show, counter to expectations, that placodal delamination does not reflect neural crest delamination but is a distinct process. Crucially, we find that placodal delamination does not involve the assumption of a mesenchymal shape by the emigrating cells, and that the placodes do not express molecules associated with EMT. Furthermore, we show that the placodal epithelium is pseudostratified, mitotically quiescent and that when cell division does occur it is apically located.

MATERIALS AND METHODS

Immunohistochemistry

Fertile hen's eggs were incubated at 38°C in humidified atmosphere to required stages (HH st) (Hamburger and Hamilton, 1992). Embryos were fixed in MEMFA overnight at 4°C. Whole-mount antibody staining was carried out as described previously (Begbie et al., 1999). Primary antibodies used were: mouse anti-neurofilament medium chain at 1:10000 (RMO-270, Zymed); rabbit anti-laminin at 1:100 (Sigma); mouse anti- β -catenin at 1:100 (Santa Cruz); mouse anti-Islet 1/2 at 1:1000 (Developmental Studies Hybridoma Bank); rabbit anti-phosphohistone H3 at 1:500 (Upstate). Secondary antibodies were Alexa 488-conjugated anti-mouse IgG and Alexa 568-conjugated anti-rabbit IgG used at 1:1000 (Molecular Probes).

In situ hybridization

Whole-mount in situ hybridization was carried out as described previously (Henrique et al., 1995).

BrdU labeling

BrdU (10 mg/ml) was applied to the surface of the embryo in ovo for 15 minutes at 37°C. Embryos were processed for whole-mount in situ hybridization with *Phox2a*. Following color development, embryos were processed for BrdU (anti-BrdU, 1:50, Biosciences Products) and cryosectioned at 10 μ m.

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Electron microscopy

Embryos were processed and analyzed for transmission electron microscopy (TEM) as previously described (Quinlan et al., 2004).

GFP electroporation

Electroporation was used to introduce chick β -actin GFP in ovo into presumptive placodal ectoderm at st15 using 4×5 millisecond pulses of 20V, or into mesencephalic crest at 9 somites using 5×5 millisecond pulses of 8V.

RESULTS AND DISCUSSION

Placodal neuroblasts emerge from a break in the basal lamina underlying distinctive placodal epithelium

Neurogenic placodes are focal thickenings of the cranial ectoderm in which neuronal cells are born, as can be readily visualized by combined immunostaining using an anti-neurofilament medium chain (NFM) antibody and an anti- β -catenin antibody (Fig. 1A). Such staining identified a number of important aspects of the morphology of the cranial ectoderm. Firstly, it was clear that the organization of the placodal ectoderm differs from that of the adjacent ectoderm. The placodal ectoderm appeared as a region many cells thick, whereas the adjacent surface ectoderm was squamosal. Secondly, the localization of the β -catenin staining in the placodal epithelium was distinct from that in the non-placodal epithelium. In non-placodal ectoderm, β -catenin was localized to the cell membrane and there are discrete regions where β -catenin levels were high (Fig. 1C). In the placodal epithelium, the β -catenin staining was uniform throughout the cells (Fig. 1D).

Also notable is that whereas the basal lamina was continuous beneath the non-placodal ectoderm (Fig. 1E), it was absent beneath the placode itself. In cross-section, neuroblasts can be seen emigrating from the placode from a region showing a lack of laminin immunoreactivity (Fig. 1F-H). This strongly suggests that there is specific breakdown of the basal lamina at the site of neuroblast exit from the epithelium. That a breach in the basal lamina is seen in both placodal and neural crest delamination is to be expected as both

processes involve the release of cells into the surrounding environment. It also suggests that the processes might share some of the same molecular mechanisms.

Delamination of placodal neuroblasts is distinct from neural crest delamination

The molecular control of neural crest cell production has been well studied (Morales et al., 2005). These studies have indicated roles for FoxD3, Ap2 and members of the SoxD and SoxE families of transcription factors. In situ analysis shows no expression of these genes in the placodal epithelium (Perez-Alcala et al., 2004; Veitch et al., 1999). However, these genes are associated with neural crest induction in addition to delamination. The process of delamination specifically has been shown to involve Snail-family zinc-finger transcription factors (Nieto, 2002) and members of the Rho subfamily of GTPases (Liu and Jessell, 1998).

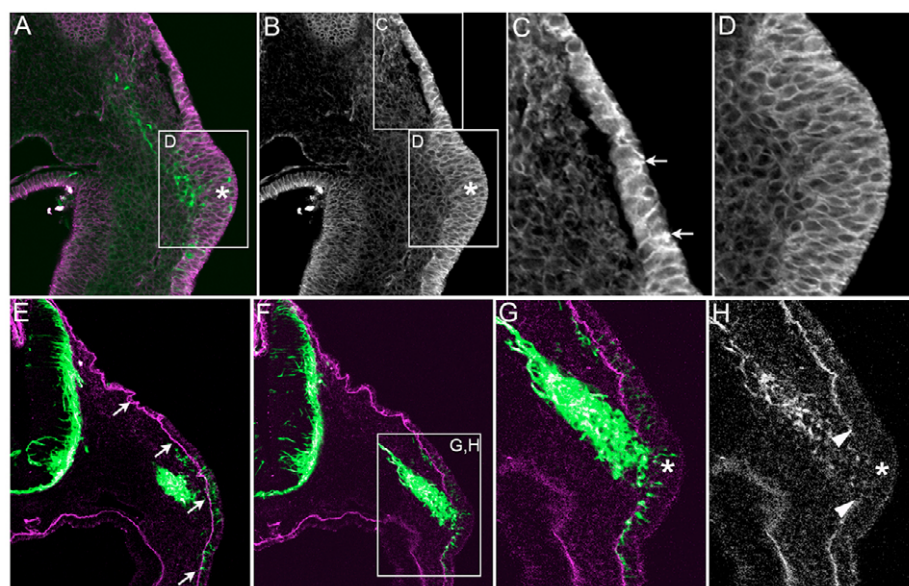
RhoB

In the trunk, *RhoB* has been shown to be required for neural crest cell delamination (Liu and Jessell, 1998). Correspondingly, our in situ analysis showed a high level of *RhoB* expression in neural crest cells exiting the neural tube in the trunk (Fig. 2C). Analysis of the head in the same embryo showed that there is pronounced *RhoB* expression within neural crest cells that adhere to the hindbrain (Fig. 2A,B). By comparison, it was clear that *RhoB* expression was not elevated in the epibranchial placodes (Fig. 2B), suggesting that *RhoB* is not upregulated in placodal delamination. Within the Rho family of GTPases, *RhoB* forms a subfamily with *RhoA* and *RhoC* (Wennerberg and Der, 2004). We therefore also analyzed the expression of *RhoA* and *RhoC*, and found that they were likewise not expressed in the placodes (data not shown).

Snail/Snail2

In neural crest delamination, *Snail2* (formerly known as *Slug*) is the key family member required in the chick and replaced by *Snail* in the mouse. As with *RhoB*, we analyzed expression of *Snail2* at st16/17 when placodal cells are actively delaminating. Where neural

Fig. 1. Placodal neuroblasts exit the characteristic placodal ectoderm through an underlying breach in the basal lamina. (A-D) Transverse confocal section of st17 chick embryo. Boxed regions in A and B are shown at higher magnification in C and D. (A,B) Characteristic morphology of thickened epithelium at the site of neuronal production (lower boxed region). (A) NFM, green; β -catenin, magenta. (B) β -catenin staining alone. (C) Non-placodal ectoderm. β -catenin is localized to the cell membrane (arrows). (D) Placodal ectoderm. β -catenin is not specifically localized and epithelium appears to consist of multiple cell layers. (E-H) Transverse confocal section of st17 chick embryo. NFM, green; laminin, magenta. (E) Non-placodal ectoderm, anterior to placode. Basal lamina intact beneath the ectoderm for the whole dorsoventral extent of embryo (arrows). (F) Placodal ectoderm showing thickened morphology and NFM-positive cells leaving the epithelium. The boxed region is shown at higher magnification in G,H. (G) Where the neuronal cells exit, there is a breach in the basal lamina. This is seen more clearly when laminin is shown alone (H); the extent of the breach is indicated by arrowheads. Laminin is also seen in neuronal cells as they migrate away. *, placodal epithelium.



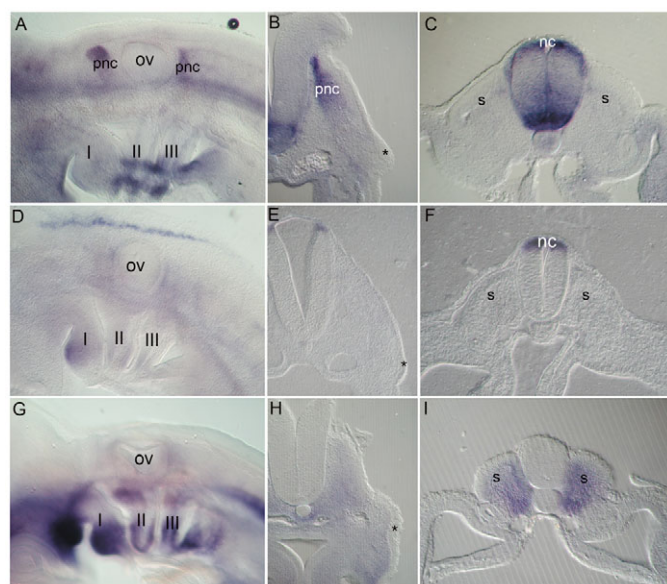


Fig. 2. Placodes do not express molecules associated with EMT.

(A-C) RhoB expression in chick at st18. (A,B) Post-migratory neural crest cells remaining in contact with hindbrain show high levels of RhoB expression. High-level expression is also seen in neural crest cells in the trunk (C), but expression is not specifically elevated in placode (B). (D-F) Snail2 expression at st17. There is no expression in the placode (E); however, a transverse section of trunk shows expression in the neural crest progenitors, as expected (F). (G-I) Snail expression at st18. Expression is seen in cranial mesoderm including post-migratory crest (G,H). No expression of Snail is detected in cranial ectoderm (H) or neural crest progenitors (I). (A,D,G) Lateral view of pharyngeal region ($\times 6$); (B,E,H) Transverse section through placode ($\times 16$); (C,F,I) Transverse section at hindlimb level ($\times 16$). nc, neural crest; ov, otic vesicle; pnc, post-migratory neural crest; I, II, III refer to the pharyngeal arches; *, placodal epithelium; s, somite.

crest cells were being produced in the trunk, expression was seen in the dorsal aspect of the neural tube (Fig. 2F). However, expression was confined to a small population of cells in the hindbrain and was absent from the placodal epithelium (Fig. 2D,E). Analysis of *Snail* showed an expected lack of expression in the presumptive trunk neural crest (Fig. 2I). Expression was seen throughout the cranial region, including the post-migratory neural crest; however, it was absent from the ectoderm at both placodal and non-placodal levels (Fig. 2G,H).

Comparison of neural crest cell delamination with other incidences of EMT (e.g. cancer cell metastases) shows that Snail and Rho GTPases are involved in organizing the changing morphology that accompanies cell delamination (Savagner, 2001; Thiery, 2002; Tucker, 2004). The lack of association of these molecules with placodal delamination suggests that cells leaving the placode do not go through an EMT.

Placodal delamination involves the release of neuronal cells

It has long been assumed that cells leave neurogenic placodes as mesenchymal cells (McCabe et al., 2004; Schlosser, 2006; Webb and Noden, 1993). Therefore, to support the molecular data we went on to analyze the cellular morphology of placodal cells within the epithelium and as they exit. In longitudinal section through the pharyngeal arches at HH st17, the thickened placodal epithelium and

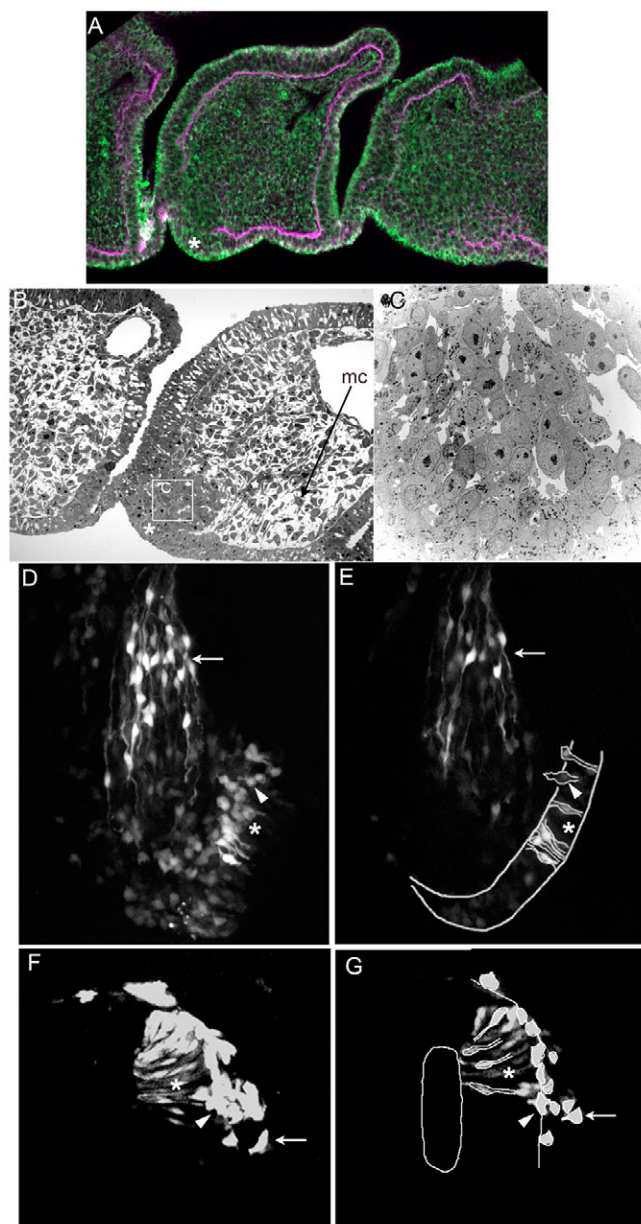


Fig. 3. Placodes resemble neuroepithelium. (A) Coronal confocal section through the third and fourth pharyngeal arches demonstrating the break in the basal lamina and the position of the placode (for comparison with B). β -catenin, green; laminin, magenta.

(B) Longitudinal semi-thin section showing that the mesenchymal morphology of surrounding neural crest cells (mc, mesenchymal cells) is distinct from the compact morphology of cells leaving the placode (*).

(C) TEM of the boxed region from B showing the round cell morphology of cells leaving the placode. (D,E) GFP labeling of placodal ectoderm and resulting neurons indicating the pseudostratified morphology of the placode. (D) Transverse confocal section showing the whole z-stack. (E) Single optical slice with the outline of labeled cells within placodal ectoderm highlighted schematically. Arrow, neurons forming ganglion; arrowhead, neuroblast leaving placode; *, placodal epithelium. (F,G) GFP labeling of mesencephalon and emigrating neural crest. (F) Transverse confocal section showing the whole z-stack. (G) Single optical slice with the border of the neural tube and the outline of labeled cells highlighted schematically. Arrow, migrating neural crest cells; arrowhead, neural crest cell with mesenchymal morphology leaving the neuroepithelium; *, neuroepithelium.

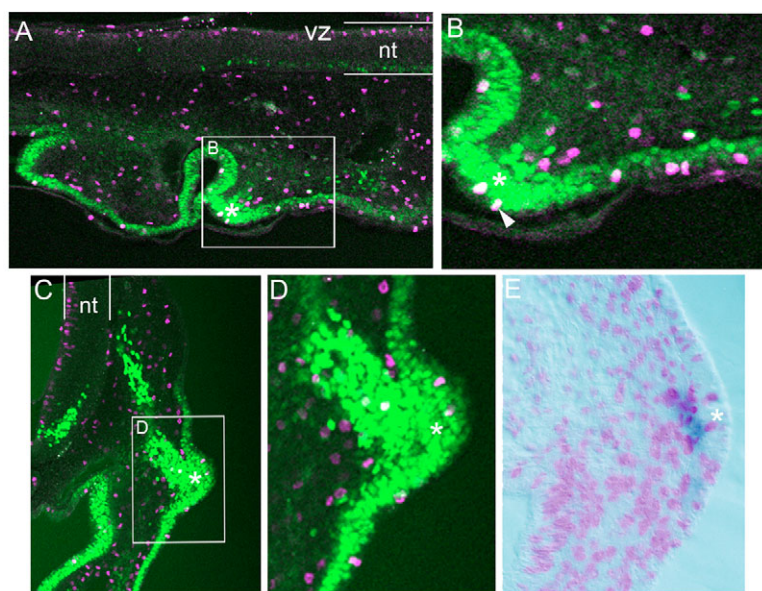


Fig. 4. Cell division occurs at the apical surface of the placodal epithelium. (A,B) Coronal confocal section through the pharyngeal arch region at st16. Islet 1/2, green; PH3, magenta. The boxed region is shown at higher magnification in B. Division occurs at the apical surface of the placode (arrowhead). Proliferation is reduced in the placode as compared with the ventricular zone (VZ) of neural tube (pial and ventricular surfaces of neural tube indicated by lines). (C,D) Transverse confocal section through the petrosal placode at st18. Islet 1/2, green; PH3, magenta. The boxed region is shown at higher magnification in D. Little division is seen within the placodal epithelium and where it does occur it is restricted to the apical surface of the placode. (E) Transverse section through the geniculate placode at st18. BrdU, magenta; Phox2a, blue. BrdU labeling confirms that there is little division in the placode and no accumulation of BrdU-positive cells at the point of exit of the placodal cells. *, placodal epithelium; nt, neural tube.

underlying breach in basal lamina were clearly visible (Fig. 3A). In an equivalent semi-thin section, the characteristic thickened placodal epithelium could be seen adjacent to the endodermal pouch (Fig. 3B). A clear distinction could be made between the mesenchymal neural crest cells filling the pharyngeal arch and the close-packed cells emerging from this placodal epithelium (Fig. 3B). Electron microscopy at this level showed that the cells emerging from the placode had a distinctively neuronal morphology, with a round cell body and large nucleus consistent with their migration as neuroblasts (Fig. 3C). Thus, the placodal cells do not assume a mesenchymal morphology and cannot be said to be undergoing EMT.

To reveal the morphology of cells within the placodal epithelium and exiting from it, we used electroporation to target GFP to a small number of cells in the placode. Confocal analysis generated a stack of images as shown in Fig. 3D. Individual optical sections were then analyzed to determine the morphology of labeled cells (Fig. 3E). The schematic in Fig. 3E shows the extent of the placodal epithelium. It visibly assumes a pseudostratified appearance, resembling the neuroepithelium rather than consisting of multiple cell layers. We also observed the morphology of cells emerging from the placode (Fig. 3D,E), and again these cells had a distinctly neuronal appearance. This can be compared with a similar GFP electroporation into neural crest cells prior to delamination (Fig. 3F,G). The schematic in Fig. 3G shows the pseudostratified appearance of the neuroepithelium within the mesencephalon, and the morphology of the neural crest cells as they exit. These cells have a mesenchymal appearance (Fig. 3G).

The pseudostratified appearance of the placodal ectoderm suggests that we could compare the placode with the germinal neuroepithelium. Here, a distinctive feature was that cell division occurred at the ventricular surface. Double immunostaining with anti-phosphohistone H3 (PH3) and anti-Islet 1/2 labels cells undergoing division in the context of the epithelium and neuroblasts migrating away from the placode. Confocal analyses of sections showed that where division does occur, it is restricted to the apical surface of the epithelium (Fig. 4A-D); this can be compared with the ventricular zone (VZ) of the neural tube in the same section (Fig. 4A,C). In the neural tube, release of cells from the VZ is usually associated with cell division. Analysis of later placodal stages using both PH3 and BrdU showed, however, that compared with the VZ,

and with the surrounding ectoderm, the placode is mitotically quiescent (Fig. 4C-E). Furthermore, neurons migrating away from the VZ are generally post-mitotic, whereas the cells that leave the placode are mitotically active (Begbie et al., 2002). The absence of BrdU-positive cells at the point of delamination (Fig. 4E) suggests that unlike neural crest cell delamination, the exit of placodal cells from the epithelium is not associated with S phase (Burstyn-Cohen and Kalcheim, 2002).

Our results demonstrate that the delamination of neuronal cells from the neurogenic placodes differs from neural crest delamination. Thus, whereas the generation of neural crest involves an EMT, this is not so for the release of cells from the placodes. This highlights the importance of considering the accuracy of definitions: neural crest cell emigration is often considered synonymously with delamination, and is cited as a parallel example in situations of cells leaving an epithelium. However, for example in liver formation (Bort et al., 2006), this might not be accurate.

The observation that the cells leaving the placodes do not undergo an EMT might in part reflect the fact that cells are produced from the placodes over a protracted period: in the geniculate placode, neurogenesis is first seen at st13 and is still ongoing at st20, a period of ~30 hours (Begbie et al., 2002). By contrast, the generation of neural crest cells from a particular axial level of the neural tube is a fairly rapid process: at the level of rhombomere 2, neural crest production initiates at st8 and is finished by st11, a period of ~14 hours (Lumsden et al., 1991).

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