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Epithelial fusion during neural tube morphogenesis

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Abstract

Adhesion and fusion of epithelial sheets marks the completion of many morphogenetic events during embryogenesis. Neural tube closure involves an epithelial fusion sequence in which the apposing neural folds adhere initially via cellular protrusions, proceed to a more stable union, and subsequently undergo remodelling of the epithelial structures to yield a separate neural tube roof plate and overlying non-neural ectoderm. Cellular protrusions comprise lamellipodia and filopodia, and studies in several different systems emphasise the critical role of RhoGTPases in their regulation. How epithelia establish initial adhesion is poorly understood but, in neurulation, may involve interactions between EphA receptors and their ephrinA ligands. Epithelial remodelling is spatially and temporally correlated with apoptosis in the dorsal neural tube midline, but experimental inhibition of this cell death does not prevent fusion and remodelling. A variety of molecular signalling systems have been implicated in the late events of morphogenesis, but genetic redundancy, for example among the integrins and laminins, makes identification of the critical players challenging. An improved understanding of epithelial fusion can provide insights into normal developmental processes, and may also indicate the mode of origin of clinically important birth defects.

Keywords

epithelium; adhesion; fusion; lamellipodia; filopodia; apoptosis; neurulation; neural tube defects; spina bifida; morphogenesis

INTRODUCTION

Epithelial fusion is a component of many morphogenetic events in which pairs of epithelial sheets become apposed and subsequently join to form a single, continuous layer. This is seen, for example, in the closing neural tube, optic fissure, eyelids, palatal shelf, tracheooesophageal foregut, cloaca and presumptive genitalia of mammals, and in the well studied process of dorsal closure in *Drosophila*. Typically, epithelia destined to form organ primordia initially undergo growth and, sometimes complex, shape change. These early events have the effect of bringing the paired structures into epithelium-to-epithelium contact, enabling the fusion process to proceed. It is important to remember that epithelial fusion does not involve cellular fusion. Instead, it represents the reorganisation of two

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adjacent, multicellular structures to form a new, unified structure that spans an anatomical, and often physiological, gap.

An important feature of epithelial fusion is its propensity to become disrupted leading to congenital malformations. Almost every morphogenetic fusion event in the developing embryo is associated with a clinically important birth defect. For example, neural tube defects (NTDs) such as anencephaly and open spina bifida result from defects of neural fold fusion, the eye defect coloboma is a disorder of optic fissure fusion, cleft palate results when the secondary palatal epithelia fail to fuse, and hypospadias results from defective fusion of the bilateral urogenital primordia. Hence, an understanding of the cellular and molecular processes that are responsible for epithelial fusions is important, not only to elucidate key events in embryonic morphogenesis but also to gain insight into the pathogenesis of clinical birth defects, and to point towards strategies for possible preventive therapies.

In this review article, we first examine the main types of cellular event that occur when paired epithelia fuse stably *in vivo*, as exemplified by closure of the mammalian neural tube. We then ask what is the evidence for the involvement of particular molecular signalling pathways, *specifically* in the epithelial fusion process? While a plethora of signalling events is known to be necessary for epithelial morphogenesis as a whole, it is important to ask which of these have actually been demonstrated to play a role in epithelial fusion, *per se*, as opposed to being involved in other, often earlier, aspects of morphogenesis.

CELLULAR MECHANISMS OF EPITHELIAL FUSION

Figure 1 shows the typical sequence of an epithelial fusion event during morphogenesis, as illustrated by mouse spinal neural tube closure. Bending in the ventral midline and at dorsolateral hinge points brings the neural folds towards each other dorsally (Figure 1A). Apical protrusions from leading edge cells then make initial contacts across the space between the folds (Figure 1B) and epithelial ‘adhesion’ is achieved, with the neural fold tips becoming tightly apposed (Figure 1C). Remodelling occurs so that the original epithelial continuity between non-neural and neural ectoderm on each side is lost, and new epithelial continuity is established. This creates a neural tube roof overlain by a continuous layer of non-neural ectoderm (Figure 1D). In the discussion that follows, we concentrate on the events depicted in Figure 1B and C, namely the mechanisms by which epithelial contact and ‘adhesion’ are established, and the nature of the remodelling events that complete the epithelial fusion process.

Cellular protrusions emanate from leading edge epithelial cells

The process of epithelial fusion has long been known to involve cellular protrusions emanating from the leading edges of the apposing epithelial structures. Since the 1970s, structures resembling lamellipodia, originally termed “ruffles”, have been observed, for example protruding from the tips of the closing neural folds in scanning electron microscope (SEM) images of neurulation stage mouse and hamster embryos (Waterman, 1976). Transmission electron microscopy (TEM) of hindbrain neural tube closure indicated the existence of specialised ‘flat’ cells at the neural fold tips, with ruffles emerging from between these cells and the adjacent surface ectoderm (Geelen and Langman, 1979). However, other levels of the body axis gave a different appearance with, for example, the midbrain showing initial contact between apposing surface ectoderm cells and the forebrain showing neuroepithelial contact. Neither region was found to exhibit ‘flat’ cells raising questions about the cell type of origin of ruffles at different axial levels (Geelen and Langman, 1979). The typical appearance of epithelial ruffles at the spinal neural folds tips of mouse neural folds is shown in Figure 2.

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Other developmental systems similarly exhibit a strong association between ultrastructural cellular protrusions and epithelial fusion. For example, the medial edge of the palatal shelves exhibit abundant filopodia prior to fusion, a phenomenon that is absent from mice null for transforming growth factor (TGF) β3, in which cleft palate is a characteristic phenotype (Taya et al., 1999). In *Drosophila*, the epithelia mediating dorsal closure also exhibit filopodial and lamellipodial protrusions which appear to act by zippering actin cables of the leading epithelial edge together (Jacinto et al., 2000; Wood et al., 2002).

Recently, the technique of live cellular imaging has been applied to mouse neural tube closure, focusing on the closing hindbrain region in intact, cultured embryos. This work confirmed the presence of filopodia-like extensions emanating from the non-neural ectoderm and making contact across the midline (Pyrgaki et al., 2010). Moreover, mice that are genetically null for members of the Ena/VASP family, which are involved in regulation of filopodia and lamellipodia, have been found to develop exencephaly (Menzies et al., 2004; Furman et al., 2007).

Epithelial adhesion

Compared with the cellular protrusion phase, much less is known about the ‘adhesive’ stage of epithelial fusion. Indeed, it is not even clear whether epithelium-to-epithelium adhesion exists as a distinct event in the morphogenetic sequence. An alternative view might be that, in the case of the neural tube, it is purely the forces of epithelial bending and apposition that keep the neural folds ‘pressed’ together physically until the remodelling phase has progressed sufficiently to achieve ‘bridging’, and then stable union, across the midline. Nevertheless, the predominant view would be that there is a likely role for molecular adhesion between pairs of fusing epithelia. This might be set up initially by the interacting cellular protrusions, and then subsequently reinforced by the action of one or more cell-cell, or cell-matrix adhesion systems. It will be important, in future research, to develop experimental assays for epithelial adhesion, in order to enable evaluation of different molecular regulatory pathways in this process.

Tissue remodelling during epithelial fusion

The requirement for breakage and reconnection of the epithelia during the fusion process is undoubted, and yet the mechanism of remodelling is unclear. The overwhelming focus has been on programmed cell death – apoptosis – as the likely key cellular basis of this element of the epithelial fusion sequence, and much circumstantial evidence supports the idea. However, recent experimental analysis has cast serious doubt on the importance of apoptosis in epithelial remodelling.

Is apoptosis required for epithelial remodelling?—Apoptosis is a widely conserved pathway in biological systems, physiologically necessary for events such as separation of the digits during limb development, removal of juvenile structures like the Mullerian duct in male mammals, and control of neuronal cell number during target innervation (Jacobson et al., 1997). Cell death in the closing mouse neural tube was first documented in ultrastructural studies, and dying cells were subsequently found to exhibit the characteristic features of apoptosis (Schluter, 1973; Geelen and Langman, 1979; Mirkes, 2002). Recently, use of live imaging to detect apoptosis has revealed distinct types of cell death in the closing neural tube (Yamaguchi et al., 2011). Experimental evidence for a requirement of apoptosis in neurulation came from finding that neural tube closure in chick embryos fails following suppression of apoptosis, by *in ovo* treatment with the pan-caspase inhibitor z-VAD-fmk (Weil et al., 1997). In mice, several knockout strains exhibit alterations in the abundance of apoptotic cells during development of NTDs. While the majority show increased cell death, loss of function of the pro-apoptotic genes *Apaf1* and *Casp3* both produce diminished or

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absent apoptosis and yet cranial NTDs are also observed (Cecconi et al., 1998; Leonard et al., 2002). However, exposure of mouse embryos to the apoptotic inhibitors zVAD-fmk (a caspase inhibitor) or pifithrin-α (an inhibitor of p53) throughout neurulation does not prevent cranial or spinal neural tube closure, despite an almost complete lack of apoptotic cells in the embryo (Massa et al., 2009). In particular, the remodelling of the neuroepithelium and non-neural ectoderm occurs in the absence of apoptosis. Hence, it seems unlikely that programmed cell death is a specific requirement for tissue remodelling during epithelial fusion. It remains unclear why NTDs occur in the *Apaf1* and *Casp3* mutants, although additional defects resulting from constitutional lack of apoptosis may be responsible.

Why do cells die at sites of tissue remodelling?—The mechanisms by which apoptotic cells become specifically associated with the site of neural tube closure may involve the process of anoikis, in which cells that lose cell-cell or cell-matrix adhesion enter upon the apoptotic pathway. The extracellular matrix is known to become disrupted during tissue remodelling at the completion of neural tube closure (Hoving et al., 1990), providing a likely cause for cells to enter anoikis. There is also evidence for a role of altered cell adhesion leading to anoikis at the site of neural tube closure. The Nf2 tumour suppressor (also called Merlin) regulates cell-cell adhesion during tissue fusion, by promoting the assembly and maintenance of apico-lateral junctional complexes. Embryos mosaic for deletion of Merlin exhibit fusion defects in a number of organs, including brain, heart, eye and palate (McLaughlin et al., 2007). These malformations derive from ectopic cellular detachment during tissue fusion, owing to failure to maintain apico-lateral junctional complexes. In severely affected Merlin mutants, apoptosis is increased more than 30-fold at the neural fold tips where ectopic detachment is particularly marked. Hence, only epithelial cells that maintain stable cell-cell contacts appear to survive through morphogenetic tissue remodelling.

Radial versus directional propagation of epithelial fusion

So far, we have considered tissue fusion only as a three-dimensional process. However, there is often a fourth, temporal, dimension as well, and it is important to consider this aspect when comparing different morphogenetic systems.

Radial fusion events—Some tissue fusions are essentially radial, in that they occur simultaneously around the circumference or along the length of an opening. While radial fusion is not a feature of mammalian neural tube closure, it does occur in events such as mouse lens placode fusion and dorsal closure in *Drosophila*. There are similarities between these events and epidermal wound healing, which is probably the most intensively studied tissue fusion event (although distinct in that it does not occur during undisturbed development). A common feature of ‘radial’ tissue fusions is the presence of a circumferential actin cable that encircles the closing gap and appears to ‘tighten’, drawing the edges together (Jacinto et al., 2002). However, once the tissue edges are apposed, the processes of adhesion and remodelling are similar to those observed in directionally propagating events. For example, both *Drosophila* dorsal closure and mammalian eyelid closure are characterised by cellular protrusions immediately prior to fusion, and programmed cell death at the fusion site (Martin and Parkhurst, 2004).

Directionally propagating fusion events—Fusion events that occur during formation of elongated organs often exhibit directionally propagating fusion. This is exemplified by neural tube closure which, in mammals, occurs in a piecemeal, multisite manner so that the completed neural tube is progressively assembled through a series of directionally propagated closure events (Copp et al., 2003). Other organs also exhibit directional closure:

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e.g. optic fissure closure, palatal closure and tracheooesophageal fusion/separation. In each of these events, fusion begins at a particular site and then propagates along the organ primordium.

Mouse neural tube closure is often likened to a zip fastener that grows as it travels along the body axis (Van Straaten et al., 1997). This raises the question of whether sequential events of epithelial fusion are initiated in response to the immediately preceding event. It may be mechanically 'easier' to continue closure than to begin the process. Consistent with this idea is the observation that, once arrested, directional closure along the spine of the mouse embryo does not appear able to re-start. Hence, spina bifida in different mouse genetic models exhibits differing rostral extents, depending on the stage at which closure arrested. In contrast, the caudal limit of spina bifida is relatively uniform between models, corresponding to the site at which primary neurulation normally finishes, and secondary neurulation begins, in the upper sacral region (Copp and Brook, 1989). It remains to be determined how the wave of zippering along the spinal region is propagated and the mechanics of this process.

MOLECULAR BASIS OF EPITHELIAL FUSION EVENTS

Rho-GTPases

The cellular protrusions that make first contact during fusion of the neural folds resemble lamellipodia and filopodia, as studied extensively in cultured fibroblasts. Lamellipodia are broad, sheet-like cell protrusions containing a branched network of actin filaments that grow at their barbed ends. In contrast, filopodia are thin, spike-like protrusions with an actin filament core (Ridley, 2011). The small GTPases, Rac and Cdc42, play key roles in regulating these cellular protrusions, with Rac traditionally considered to induce formation of lamellipodia and membrane ruffling, and Cdc42 associated with formation of filopodia (Hall and Nobes, 2000). However, this is likely to be an over-simplification as both Rac and Cdc42 have more recently been implicated in the formation of both types of protrusion, depending on cellular context. The GTPases regulate polymerization of actin microfilaments at the leading margin of the cell via activation of the Arp2/3 complex (Vasioukhin et al., 2000), with involvement of a host of interacting proteins that provide specificity and determine the precise nature of the protrusions: lamellipodia or filopodia. For example, members of the N-WASP family have particularly been implicated in lamellipodium formation, while members of the formin family appear particularly important in filopodium formation (Ridley, 2011).

Experimental evidence for a role of RhoGTPases in regulating cellular protrusions during epithelial fusion comes from several morphogenetic systems. Perhaps best understood is *Drosophila* dorsal closure, in which the assembly of cellular protrusions is blocked by inhibition of either Cdc42 or Rac1, the latter acting upstream of JNK signalling (Jacinto et al., 2000; Shimizu et al., 2005; Woolner et al., 2005). Conversely, activation of Rho1 signalling is required for the assembly of the actin cable that acts as a purse string within the leading edge, narrowing the dorsal opening as it contracts (Jacinto et al., 2002). This role of Rho activation in actin cable assembly is conserved in mammals: in the Rho-associated kinase (ROCK)-1 knockout mouse, open eyelids and failure of body wall closure result from disruption of the actin cable that normally encircles the closing eyelid leading edge (Shimizu et al., 2005).

There have been few experimental assessments to date of RhoGTPase-mediated regulation of cell protrusion during neural fold fusion. This is part due to the pre-neurulation lethality of both *Rac1* and *Cdc42* knockout mice (Sugihara et al., 1998; Chen et al., 2000). Recently, however, Camerer et al. (2010) conditionally ablated *Rac1* function in the non-neural

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ectoderm using *Grhl3*Cre, and described failure of both cranial and spinal neural tube closure in a proportion of embryos. While it was not determined whether the failure of neural tube closure was mediated via disturbance of epithelial fusion, the possibility arises that Rac1 is indeed required for cell protrusive activity during neural fold fusion.

EphA receptor-ephrin A interactions

Eph receptors are integral membrane proteins that interact with ephrinA ligands linked to the plasma membrane by a glycophosphatidylinositol (GPI) anchor. When activated, both receptor and ligand induce intracellular signalling, described as ‘forward’ and ‘reverse’ signalling (Murai and Pasquale, 2003). Eph-ephrin interactions have been studied during adhesion and fusion in a number of morphogenetic systems, including neurulation (Holmberg et al., 2000), palatal closure (Risley et al., 2009), cloacal fusion (Dravis et al., 2004), fusion of the coronal skull suture (Merrill et al., 2006) and formation of the atrioventricular valves and septa (Stephen et al., 2007).

Neural tube closure in the cranial region requires ephrinA5 and EphA7, since null mutants for either gene exhibit cranial NTDs (Holmberg et al., 2000). It was reported that the cranial neural folds achieve full elevation, but fail to close, arguing for a defect of a late event, perhaps epithelial fusion. In the spinal region, while five EphAs (A1-A5) are expressed in the region of neural fold closure, EphA2 is present specifically at the tips of the neural folds just before closure occurs, arguing for a key role of this receptor in spinal epithelial fusion (Abdul-Aziz et al., 2009). EphA2 expression can also be detected on the lamellipodia-like protrusions emanating from the neural folds, suggesting that EphA-ephrinA interactions could form part of an early adhesion event in fold fusion (Abdul-Aziz et al., 2009).

Experimental evidence for a role of EphA-ephrinA interactions in mediating spinal neural fold closure has come from manipulation of whole cultured mouse embryos. Cleavage of GPI-anchored molecules, including ephrinAs, from the embryonic cell surface results in delay of spinal neural tube closure. Moreover, injection of EphA fusion proteins intra-amniotically into cultured embryos, which is known to specifically disrupt EphA-ephrinA interactions, delays spinal neural tube closure, without adverse effects on growth or developmental progression. Importantly, these treatments do not disturb neural plate bending or neural fold elevation, arguing for a primary effect on neural fold adhesion and/or fusion (Abdul-Aziz et al., 2009). It appears possible, therefore, that the EphA-ephrinA interaction system could underlie fusion of the mouse neural folds, perhaps via initial adhesion mediated through cellular protrusions expressing EphA receptors.

Epithelial cadherins and CAMs

It might be anticipated that the major cell-cell adhesion proteins - cadherins and cell adhesion molecules (CAMs) - would be implicated in epithelial fusion during morphogenesis, but there is rather little evidence for a specific role. N-cadherin, whose expression is restricted to the neuroepithelium, is not required for mouse neural tube closure (Radice et al., 1997), although defects in cell polarisation and convergent extension have been reported for zebrafish embryos lacking N-cadherin function (Hong and Brewster, 2006). Similarly, loss of NCAM function is compatible with normal neurulation (Cremer et al., 1994) although post-translational modification of the NCAM protein has been detected in the *splotch* (*Pax3*) mouse model of NTDs (Moase and Trasler, 1991). Direct assessment of the role of E-cadherin is hampered by the pre-neurulation lethality of null embryos (Larue et al., 1994). Recently, *Grhl2* loss of function mouse models have been generated which display exencephaly, craniofacial defects and spina bifida (Rifat et al., 2010; Werth et al., 2010; Brouns et al., 2011). In another, hypomorphic, *Grhl2* model spina bifida is absent but longer survival of mutants reveals the occurrence of thoracoabdominoschisis (Pyrgaki et al.,

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2011). Grhl2 was found to directly regulate E-cadherin in epithelial cells (Werth et al., 2010), raising the possibility that misregulated E-cadherin could play a role in the phenotype of *Grhl2* mutants (Werth et al., 2010; Pyrgaki et al., 2011).

Integrins and the extracellular matrix

Integrins are major transmembrane adhesion molecules that mediate cellular interactions with the extracellular matrix (ECM), particularly at focal adhesions. Integrins consist of α and β subunits that, upon binding ECM components, activate non-receptor kinases (such as Src family kinases) which transmit signals intracellularly to the actin cytoskeleton (Hynes, 2002). In this way, cell shape and motility - key events in morphogenesis - can be influenced directly by alterations in ECM composition, integrin expression, or both.

Determining the role of integrins in epithelial fusion events has been hampered by the diversity of α and β chains, which generates significant functional redundancy. For example, simultaneous inactivation of all the αv integrins in mice, while a potent cause of placental defects, is compatible with survival to birth of a proportion of mutants. Cleft palate is the major developmental defect in these mice (Bader et al., 1998). A role for integrin signalling during neural tube closure is suggested by the finding of NTDs in a proportion of mice lacking both α3 and α6 integrins (De Arcangelis et al., 1999). Moreover, open cranial and spinal NTDs were observed in experiments in which the signalling-competent β1A isoform was replaced genetically by an alternatively spliced form β1D, which cannot mediate mechano-transduction to establish intracellular signalling (Baudoin et al., 1998). These findings argue strongly for the importance of integrin-mediated signalling in mouse neurulation.

The ECM ligands for integrins include laminins and fibronectin. Mice lacking fibronectin undergo developmental arrest around the start of neurulation, hampering an assessment of fibronectin's role in neural tube closure (George et al., 1993). In contrast, failure of cranial neural tube closure is observed in mouse embryos lacking laminin α5 (Miner et al., 1998). Since α5-containing laminins are an abundant component of newly-formed basement membranes at the stage of neurulation (Copp et al., 2011), it seems likely that these laminins may play a key role through interaction with integrin receptors. Whether such putative integrin-laminin interactions are implicated in neuroepithelial fusion *per se* remains to be determined.

JNKs

The c-Jun NH_2-terminal kinase (JNK) subgroup of mitogen-activated protein kinases (MAPKs) have been implicated in morphogenetic events in both mouse and *Drosophila*. The HEP protein in *Drosophila* is homologous to JNK, and *hemipterous* (*hep*) mutants do not exhibit filopodial or lamellipodial protrusions, resulting in failure of dorsal closure (Glise et al., 1995). In mice, double null mutants for *Jnk1* and *Jnk2* exhibit embryonic lethality accompanied by hindbrain NTDs, with apparent dysregulation of apoptosis (Sabapathy et al., 1999; Kuan et al., 1999). Interestingly, mutants null for *Jnk1* but heterozygous for *Jnk2* ($Jnk1^{-/-}/Jnk2^{+/-}$) are viable but exhibit open eyelids and retinal coloboma, the latter reflecting failure of optic fissure closure (Weston et al., 2003). These findings confirm redundancy in JNK function during mammalian morphogenesis, and point to a key role in multiple fusion processes for the JNK pathway. In view of the findings in *Drosophila* dorsal closure, it would be informative to explore the role of *Jnk* genes in relation to formation of cellular protrusions during mammalian epithelial fusion.

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CONCLUSIONS

Epithelial fusion in several different systems, including neural tube closure, is characterised by protrusion of lamellipodial and filopodial cellular extensions. Accumulating evidence supports a molecular regulatory mechanism that involves the RhoGTPases, with Rac1 and Cdc42 serving to enhance protrusions, and RhoA stabilising actin and discouraging protrusion. While EphA-ephrinA interactions are implicated in the adhesive events that likely occur between cellular protrusions of the closing neural tube, we know little about how such adhesions are converted into a more stable epithelial union. With regard to the subsequent step of epithelial remodelling, this does not appear to require apoptosis. Other processes, for example epithelial-mesenchymal transition, may underlie the transient loss of epithelial structure before continuity is re-established as fusion is completed. In probing the molecular basis of the fusion events, there remains a difficulty in distinguishing failure of adhesion and fusion from earlier morphogenetic events, which include cell migration, cell rearrangements and epithelial shape change. Overlapping signalling pathways, with a high level of functional redundancy, make identification of the key molecular interactions challenging. Considerable technical and experimental hurdles also exist, particularly in relation to the application of live cellular imaging approaches to epithelial fusion during neurulation. The goal is to combine these emerging methodologies with the increasingly sophisticated genetic manipulation techniques, that allow conditional regulation of gene targeting or over-expression in mammalian systems. It can then be expected that the remaining mysteries of epithelial fusion will begin to be resolved.

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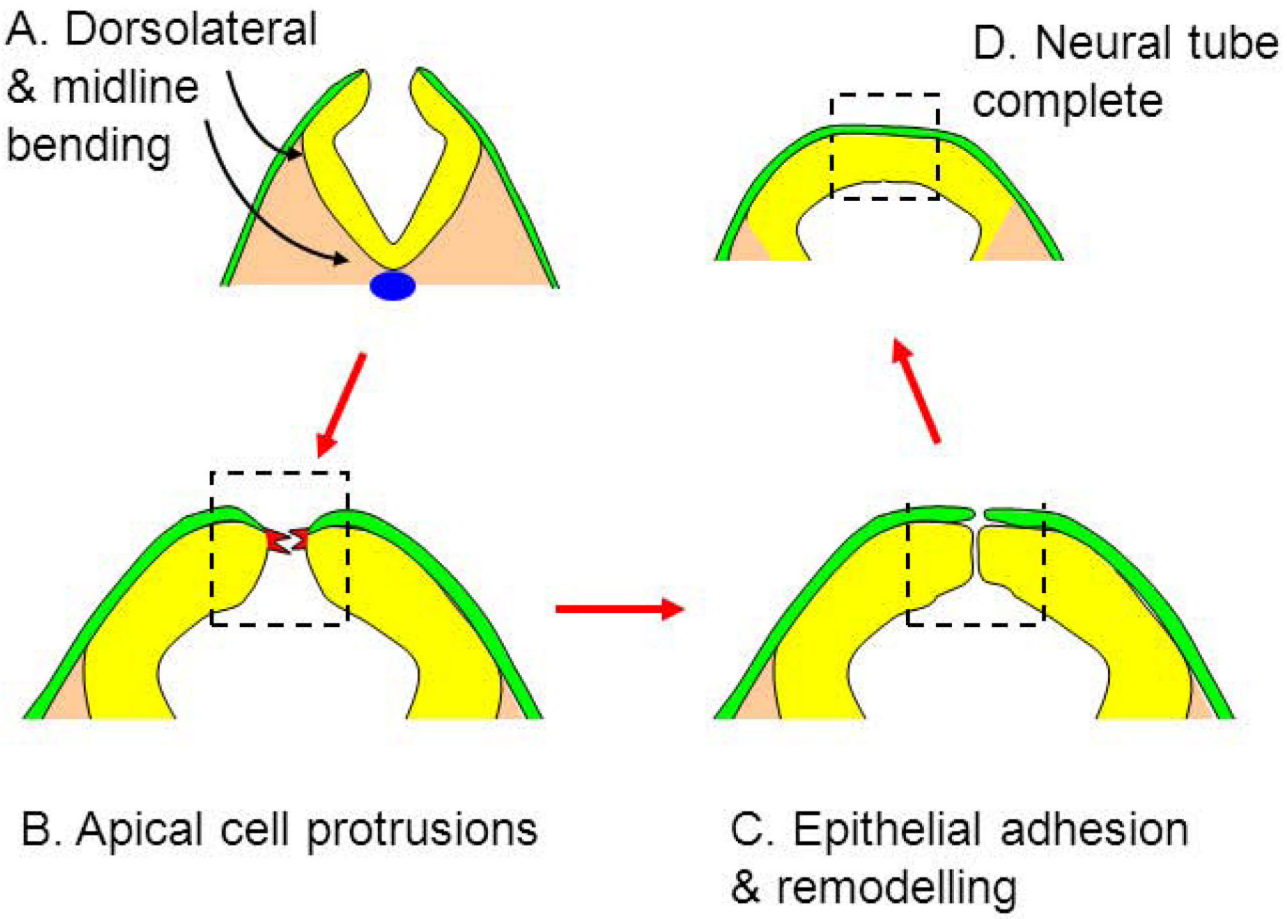

Figure 1.

Diagrammatic representation of the late events in mammalian spinal neural tube closure. (**A**) Regulated bending of the neural plate at both median (MHP) and dorsolateral (DLHP) hinge points brings the neural fold tips into apposition in the dorsal midline. (**B**) Epithelial protrusions are visible emanating from the fold tips, and making the first contact across the gap between neural folds. (**C**) Epithelial adhesion occurs together with onset of remodelling, in which the continuity of the surface ectoderm-neuroepithelium layer is broken on each side. (**D**) Epithelial continuity is re-established with formation of a continuous neural tube roof plate and overlying surface ectoderm.

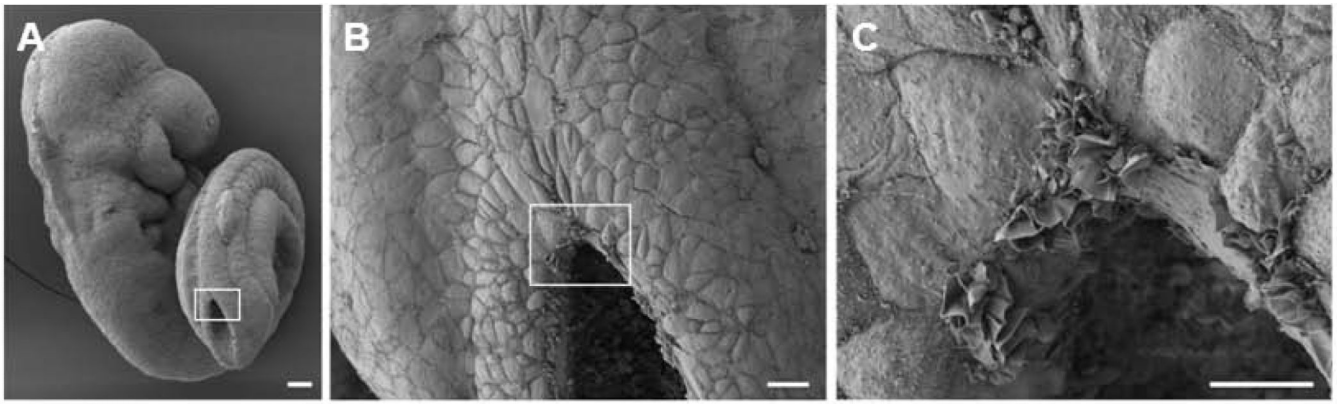

Figure 2.

Scanning electron micrographs of the dorsal aspect of a mouse embryo at embryonic day (E) 9.5. (**A**) Back view of the whole embryo. The neural tube is completely closed except for the posterior neuropore in the spinal region. Boxed area is the closure point of the neuropore, where cellular protrusions are most abundantly seen. (**B**) Enlargement of the boxed area in A. the polygonal outlines of the surface ectoderm cells are clearly visible, as are cellular protrusions emanating from the edges of the neural folds as they approach each other in the midline. (**C**) Higher magnification of the boxed area in B. The cellular protrusions can be seen as complex, three dimensional cellular lamellipodia (also called membrane 'ruffles'). Note that the lamellipodia appear to emerge from the junction between the surface ectoderm and the neural plate, which forms the inner lining of the posterior neuropore.

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