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Cell Reorganisation During Epithelial Fusion and Perforation: The Case of Ascidian Branchial Fissures

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ABSTRACT **In this study, we have analysed ultrastructurally the mechanism of epithelial fusion and perforation during the development of branchial fissures in the larva and bud of the colonial urochordate *Botryllus schlosseri*. Perforation of membranes represents an important process during embryogenesis, occurring to create communication between two separate compartments. For example, all chordate embryos share the formation of pharyngeal plates, which are constituted of apposed endodermal and ectodermal epithelia, which have the capacity to fuse and perforate. Although the process of perforation is extremely common, its cellular mechanism remains little understood in detail, because of the complexity of the structures involved. In *B. schlosseri*, two epithelial monolayers, the peribranchial and the branchial ones, with no interposed mesenchymal cells, participate in pharyngeal perforation. Blood flows in the interspace between the two cellular leaflets. Apico-lateral zonulae occludentes seal the cells of each epithelium, so that the blood compartment is separated from the environment of the peribranchial and branchial chambers; here, sea water will flow when the zooid siphons open. Stigmata primordia appear as contiguous thickened discs of palisading cells of branchial and peribranchial epithelia. The peribranchial component invaginates to contact the branchial one. Here, the basal laminae intermingle, compact, and are degraded, while the intercellular space between the two epithelia is reduced to achieve the same width as that found between the lateral membranes of adjacent cells. Cells involved in this fusion rapidly change their polarity: they acquire a new epithelial axis, because part of the adhering basal membrane becomes a new lateral surface, whereas the original lateral membranes become new apical surfaces. Before disassembling the old tight junctions and establishing communication between branchial and peribranchial chambers, cells of the stigmata rudiments form new tight junctions organised as distinct entities, so that the structural continuum of the epithelial layers is maintained throughout the time of fusion and perforation.**

Key words: asexual reproduction; cell polarity; morphogenesis; perforation; pharynx; tight junction; Urochordata

INTRODUCTION

Perforation of transient epithelia is a common developmental mechanism that occurs in many locations to create a communication between two separate chambers or between an internal chamber and the external environment. This important morphogenetic mechanism, follows the temporary fusion between specific portions of epithelial surfaces. A classic example is the formation of the pharyngeal fissures, a characteristic shared by embryos of all chordates (i.e., vertebrates, cephalochordates, and urochordates). When any endodermal pharyngeal epithelial pouch comes into proximity with the corresponding overlying ectodermal grooves, elongated membranes, known as pharyngeal closing plates, form at the sites of apposition; endodermal and ectodermal epithelia then fuse and perforate.

Disappearance of a transient membrane may result from one of several events, such as programmed cell death and/or cell rearrangements, operating singly or in combination. Programmed cell death plays key roles in morphogenesis by eradicating unneeded cells. It has been demonstrated as a mechanism of opening the oral membrane in *Rana japonica* (Watanabe et al., 1984), the urogenital and the anal membranes during cloacal morphogenesis in rat (Qi et al., 2000), the chick anal membrane (Miller et al., 1998), the cloacal membrane in development of human anorectum (Nievelstein et al., 1998), the male anterior uretra in human and mouse embryos (van der Werff et al., 2000), the epithelial plate in development of the external auditory canal in mouse (Nishizaki et al., 1998), and the operculum during anuran tadpole metamorphoses (Sasaki et al., 1983); it also participates, in association with other mechanisms, in the perforation of the chicken heart foramen secundum (Morse and Hendrix, 1980).

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Cellular rearrangements resulting in the incorporation of transient epithelial cells into adjacent epithelia is another possible mechanism for the perforation of membranes. This mechanism was suggested for the rupture of the pharyngeal closing plates (Waterman, 1985; Miller et al., 1993), oral membrane (Waterman and Balian, 1980; Waterman and Shoenwolf, 1980; Miller and Olcott, 1989) and dorsal mesocardium in chick embryo (Litke and Johnson, 1980), in the perforation of the oral membrane in hamster (Waterman, 1977) and zebrafish (Waterman and Kao, 1982), in the rupture of the closing plates in the rainbow trout (Gonzalez et al., 1996), and in the formation of the semicircular canals in teleost otocyst (Waterman and Bell, 1984). In these cases, a general sequence of events of epithelial fusion and rupture can be recognised: initial contacts between the opposed epithelia occur at several small points which are separated by regions containing mesenchymal cells and extracellular material. During this process, there is a simultaneous decrease in extracellular space, cells of the two layers interdigitate to create a single cellular layer that thins and several perforations appear owing to enlargement of the intercellular spaces and separation of adjacent cells; eventually, a canal is achieved. Also, differential growth has been suggested as a contributor to perforation. Both in the rupture of chick oral membrane (Miller and Olcott, 1989) and closing plates (Miller et al., 1993), the patterns of proliferation of epithelial cells in the perforation area show distinct domains, the adhering cells dividing at significantly lower indices than cells located around the contacting surfaces. This differential could produce either tension or compression in the sites of rupture.

Epithelial fusion and successive creation of perforations has often been described as the "remodelling" of an area of tissue. However, despite a considerable number of observations, the steps that ultimately lead to the rearrangement of adherent epithelial cells, remain unknown in detail, owing to the morphologic complexity of vertebrate transitory membranes, which are constituted of multilayered fusing epithelia, with interposed mesenchymal cells. In particular, it would be of interest to know the detail of cell reorganisation during epithelial fusion and perforation and the mechanism permitting the maintenance of separated plasmalemmal domains and the barrier between apical and basolateral compartments.

In this study, we present the cellular mechanism of epithelial fusion and perforation during the differentiation of the branchial fissures in the urochordate ascidian *Botryllus schlosseri*. The swimming tadpole larvae of ascidians have the basic body plan of chordate, possessing a notochord, a dorsal nervous system, a muscular tail and a perforated pharynx. As in vertebrates, pharyngeal openings form at the sites of apposition of the ectodermal component, the atrial epithelium, and an endodermal component, the branchial epithelium (Julin, 1904). The larva undergoes metamorphosis and develops into a sessile, filter-feeding adult form (oozooid). The adult has a conspicuous pharynx furnished with numerous gill openings and a ventral, median groove, the endostyle, homologous to the vertebrate thyroid gland (Willey, 1893). Moreover, ascidians share with vertebrates several developmental genes (Wada et al., 1996, 1998; Shimeld, 1999; Shimeld and Holland, 2000), mechanisms of morphogenesis (Manni et al., 2001), and cellular properties, such as the intercellular junctional pattern (Martinucci et al., 1988; Lane et al., 1994). Investigating the ascidian developmental dynamics, therefore, may provide us with important cues pertaining to chordate development. In particular, the fusion process can be readily analysed during the formation of branchial openings, because only two simple epithelia, respectively the peribranchial and the branchial epithelium, not separated by mesenchymal tissue, are involved. It is noteworthy that colonial species, such as *B. schlosseri*, form numerous filtering zooids (blastozooids) by asexual reproduction and the perforation of their branchial walls can be followed during the formation of branchial fissures in their buds (Berrill, 1941; Casagrande et al., 1993).

In *B. schlosseri*, in both larvae and buds, the fissures (stigmata) are arranged in several parallel series, which develop according to an anteroposterior and dorsoventral gradient, and here the sequence of events occurring during epithelial fusion can be analysed in the same individual. Our data show that branchial and peribranchial cells involved in fusion contact each other by means of their basal plasma membranes after having degraded the interposed basal laminae; this event is followed by a change of the cell polarity and complete reorganisation of their tight junctions. This happens in such a way that both the continuity of the epithelia, and the separation between the basal and luminal compartments, are maintained throughout the time of fusion and the perforation of epithelia.

RESULTS

Pharynx

The pharynx, or branchial basket, of the adult blastozooid of *Botryllus schlosseri* is a conspicuous sac perforated by rows of elliptical, ciliated stigmata which are anteroposteriorly elongated and whose basic structure was previously described (Martinucci et al., 1988) (Figs. 1, 2). Cilia generate the water currents essential for feeding and respiration: water enters the branchial chamber through the oral siphon, propelled by the stigmatal cilia, and passes into the two lateral peribranchial chambers, which join in the dorsal atrial chamber. Water then leaves the body through the atrial siphon. The wall of the pharynx is formed by two opposite epithelia, facing the branchial and peribranchial cavities, respectively, and blood flows in their interspace (Fig. 1). The stigmatal cells form a ring of seven-cells thick, inserted in the double wall, and each cell bears a single row of long cilia protruding into the

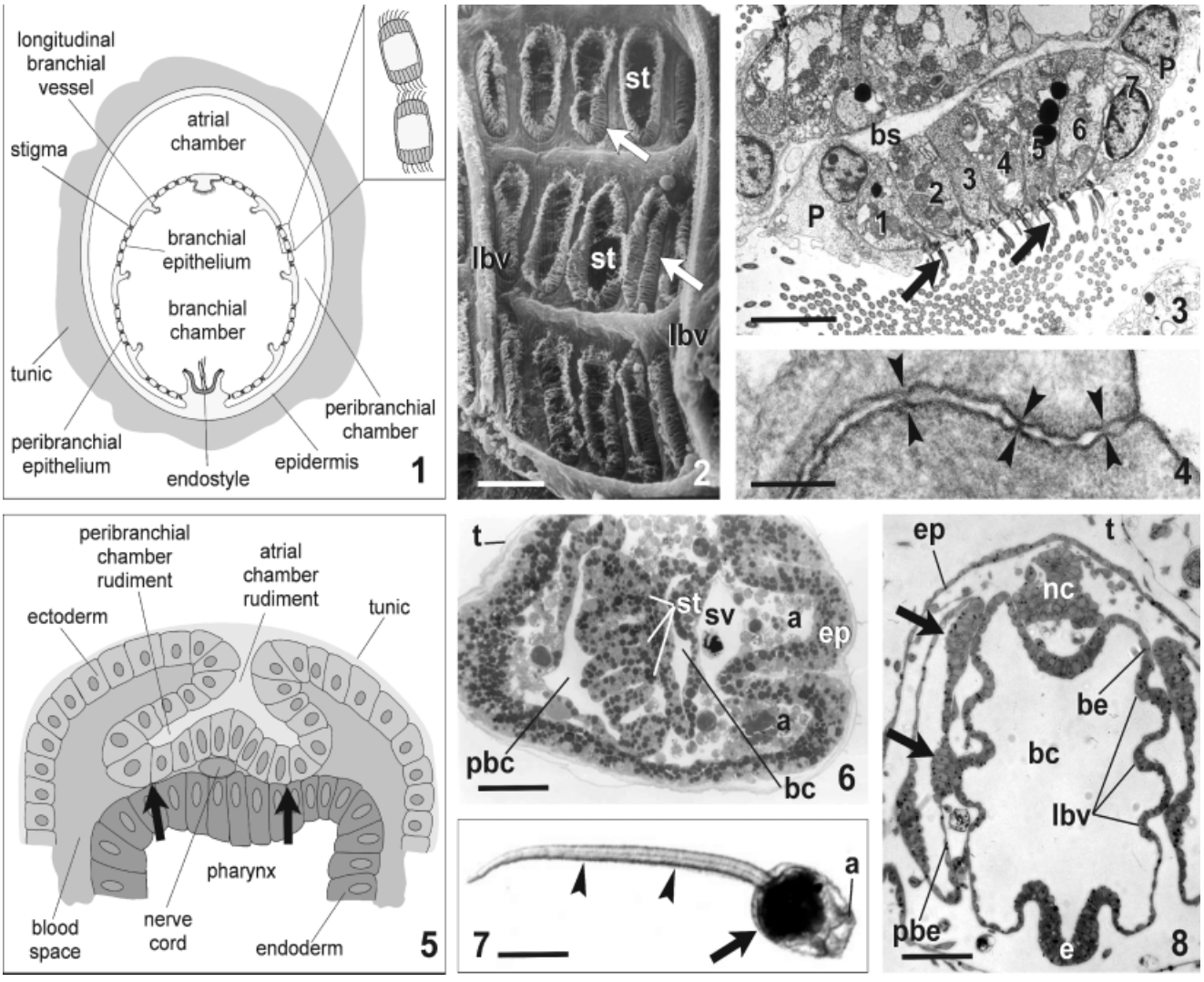


Fig. 1. Schematic transverse section of a blastozooid of *Botryllus schlosseri* at the branchial basket (pharynx) level. Stigmata are shown in detail in the inset.

Fig. 2. Scanning electron microscopy photograph of the pharynx wall of the blastozooid seen from the branchial cavity. Three rows of ciliated, elliptical stigmata (st) are visible. Arrows, stigmatal cilia; lbv, longitudinal branchial vessel. Anterior at top, posterior at bottom. Scale bar = 60 μm.

Fig. 3. Transverse section through the ciliated, seven-cell ring of a protostigma flanked by parietal cells (P). Arrows, stigmatal cilia; bs, blood space; 1–7, ciliated cells. Transmission electron microscopy. Scale bar = 4 μm.

Fig. 4. Tight junction extending between stigmatal cells. Arrowheads indicate the typical points of apposition between the two adjacent junctional membranes. Transmission electron microscopy. Scale bar = 200 nm.

Fig. 5. Schematic drawing showing a transverse section of the atrial region in an early embryo. The dorsal ectoderm is invaginated to contact the posterior part of endodermal pharynx. Arrows indicate the areas in which protostigmata will perforate. (Modified from Scott, 1934).

Fig. 6. Sagittal section of a young embryo passing through the stigmatal primordia (st) and showing the relationships among the branchial (bc) and peribranchial (pbc) chamber rudiments. a, ampullae; ep, epidermis; sv, sensory vesicle; t, tunic. Toluidine blue. Scale bar = 50 μm.

Fig. 7. Larva of *Botryllus schlosseri*. a, ampulla; arrow, larval trunk; arrowheads, tail. Scale bar = 200 μm.

Fig. 8. Transverse section of a bud through the branchial chamber (bc). Stigmatal primordia (arrows) are in the form of thickenings of the peribranchial epithelium (pbe); the primordia of the three pairs of longitudinal branchial vessels (lbv) are recognisable as evagination of the branchial epithelium (be). e, endostyle; ep, epidermis; nc, neural complex; t, tunic. Toluidine blue. Scale bar = 40 μm.

fissure and beating in coordination. Ciliated cells are flanked by thin columnar "parietal cells."

In the oozooid, four to five long protostigmata, dorsoventrally oriented, drive water from the branchial to the peribranchial chamber. These fissures are bordered by seven rows of cells, each bearing a single row of cilia accompanied by one or two rows of microvilli; ciliated cells are flanked by parietal cells (Fig. 3). Both in blastozooid and oozooid, ciliated stigmatal cells are bound to each other by extended junctional complexes in which tight (zonulae occludentes) (Fig. 4) and gap junctions are recognisable. In the blastozooid of

B. schlosseri, the presence of these types of junction was ascertained both in sections and replicas (Lane et al., 1994).

Origin of Stigmata Primordia in Embryo and Bud

In the embryo, the primitive endodermal gut is a cavity expanded anteriorly and narrowed posteriorly, hence, triangular in sagittal section; dorsally, its wall contacts the nervous system, containing the rudiment of the sensory vesicle, which then expands anteriorly to the right thereby pushing in the right side of the developing pharynx. The atrial chamber originates as a dorsal invagination of the ectoderm, which sinks into the body and bifurcates over the nervous system to form the rudiment of the peribranchial chambers. These latter descend ventrally and, on both sides, abut their anterior walls against the pharyngeal walls. In the contact area, the stigmatal primordia begin to appear (Figs. 5, 6). The gill slits will perforate and extend to form parallel fissures, which will function in the swimming larva (Fig. 7).

During asexual reproduction, the buds originate as thickenings of the peribranchial epithelium covered by the epidermis and early reach the form of a double vesicle. In the inner vesicle, two long parallel invaginations of the prospective ventral side grow to divide the original, inner vesicle into a central branchial chamber flanked by the two peribranchial chambers. Thus, for each invaginating fold, the two walls facing each other represent, respectively, the rudiments of the branchial and peribranchial epithelia. They contact each other in aligned thick zones representing the primordia of the stigmata. At the stage in which the bud heart begins beating, these primordia appear as small buttons of the peribranchial epithelium arranged in rows along the ventrodorsal axis of the branchia (Fig. 8).

The cellular mechanisms that give rise to peribranchial and branchial epithelia fusion and stigma primordial perforation are similar in both larva and bud. The data used here document stigmatal differentiation obtained mainly from observations on the latter.

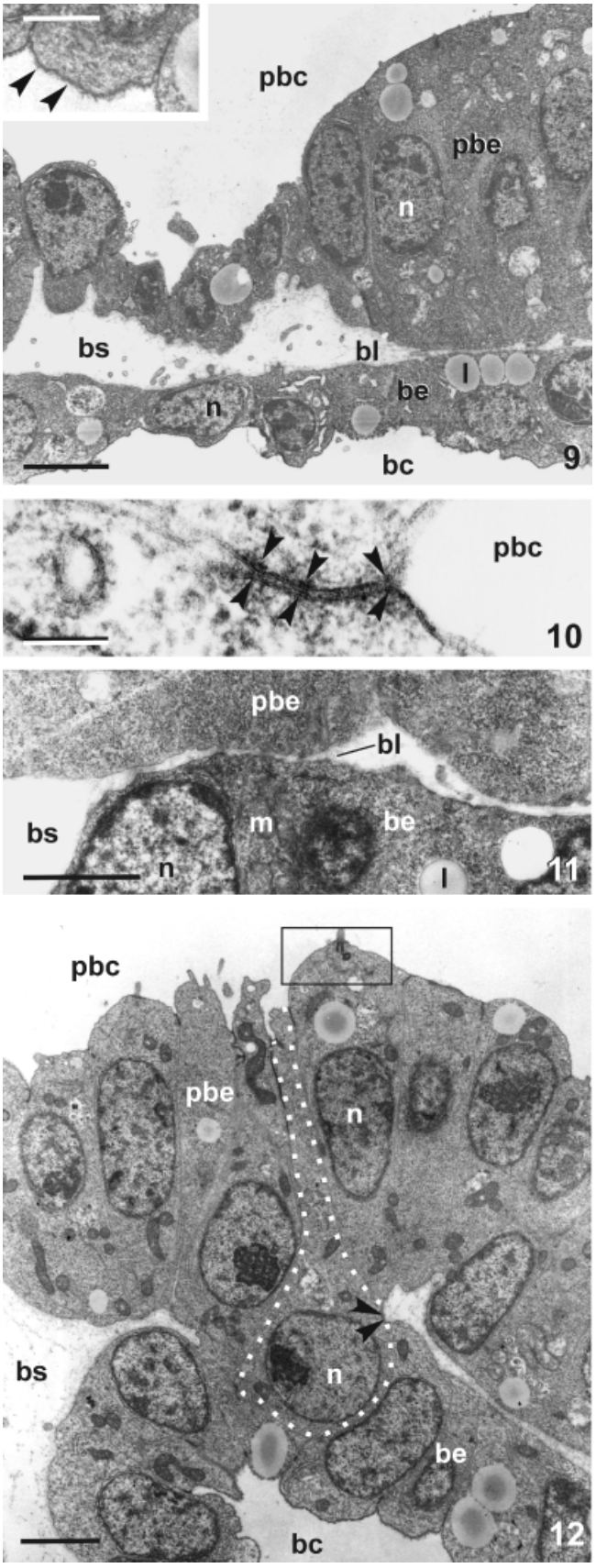
Cellular Fusion and Stigma Primordial Perforation

When stigmata primordia become recognisable, the simple branchial and peribranchial epithelia are polarised (Fig. 9): they have a thin basal lamina covering the side bathed by blood, whereas on the opposite side, dense intercellular junctions extend all around the apicolateral cell membranes. These junctions (Fig. 10), which are quite shallow, show points of fusion of the outer membrane leaflets of adjacent cells, so that they look like typical tight junctions with fibrous material on the cytoplasmic side. Moreover, the apical plasmalemma of branchial cells is covered by a thick, dense, fuzzy glycocalyx, which represents a useful marker to distinguish these cells from the peribranchial ones during the entire process of stigmata formation (inset in Fig. 9). In each primordium, peribranchial cells are thick and have a palisade appearance in transverse and sagittal sections. Close to them, the branchial cells also thicken, although not so much as the peribranchial ones (Figs. 8, 9). Mitotic figures are very frequent in the whole area of the primordium. In each primordium the two opposed thickenings have the aspect of "rosettes" and the contact between the two occurs thanks to the extensions of the peribranchial cells.

Epithelial adhesion between the two kinds of rosettes begins when the peribranchial component approaches the branchial epithelium in such a way that blood no longer flows in that area. The basal laminae of the two epithelia intermingle and compact (Fig. 11), become discontinuous at points, and finally are no longer recognisable; this process permits the fusion of the two epithelia with the formation of new cell–cell contacts between cells of the opposite layers. The intercellular space separating the original basal plasmalemmata of the two epithelia becomes reduced to a 10- to 12-nm cleft, the same space that commonly separates the lateral plasmalemmata in contiguous cells of the same epithelium (Fig. 12). Indeed, in the cells involved in the contact, most of the basal plasmalemma actually become lateral, whereas only a restricted portion conserves the original basal characteristics, remaining covered by the basal lamina (Fig. 12).

Moreover, owing to the pressure of the peribranchial cells, the branchial cells become attenuated so that their lateral membranes become very short and reduced to the areas of the tight junctional zones (Figs, 13, 14). Close to them, dense membranal areas become recognisable, as presumptive signs of new tight junctions, at the point of contact between the original basal plasmalemma of branchial and peribranchial cells. At the same time, part of the original lateral membrane of the peribranchial cells begins to acquire the features of new apical surface (Figs. 13, 14). All this shows that changes in polarity are occurring both in the branchial cells and the peribranchial cells. In this morphogenetic process the peribranchial area involved can constitute the bottom of an invaginating pit at the centre of the peribranchial thickened disc (as described by Casagrande et al., 1993), or the bottom of a solid button, whose cells extend deeply to reach the branchial cells with their basal membrane, and only later do they shorten during stigmatal fissure formation (Fig. 12). In both the cases, the branchial epithelium becomes thinner and thinner owing to the pressure of the peribranchial cells (Figs. 13, 14).

Most of the nuclei of the rudiment are elliptical, with their major axis oriented parallel to the longitudinal cell axis. However, cells that are fusing with the branchial leaflet have nuclei which change orientation, in such a way that the major nuclear axis becomes parallel to the axis connecting the narrow basal plasmalemma and the opposite plasmalemma which was lateral and is becoming apical (Fig. 13; see also Fig. 21). In addition, cytoplasmic organelles, such as the Golgi



Figures 9–12

complex, change in their distribution inside the cells demonstrating the rotation of the cell axis: initially, they are located above the nucleus on the apical area of the peribranchial cells, facing the peribranchial chamber (Fig. 15); later, they become recognisable in the new apical area of the cell, between the re-oriented nucleus and the apical surface, which eventually will border the branchial fissure.

Here, tight junctions are assembled and extend all around the new apicolateral cell membranes (Figs. 16, 17). Thus, cells involved in polarity change are provided at this stage with two types of tight junctions: the old ones, sealing peribranchial cells and branchial cells together; and the new ones, sealing together the two rings of peribranchial and branchial cells involved in fusion.

Between the new apical plasma membranes, a space, the new stigmatal fissure, begins to become evident (Fig. 17). Eventually, the old tight junctions are no longer recognisable and the stigma perforation results (Figs. 18, 19). The continuity of the epithelial monolayers is always maintained throughout the process of fusion and perforation so that no communication occurs between blood space and branchial lumen.

The cells surrounding the newly formed canal crossing the thickness of the pharyngeal wall are all of peribranchial origin and represent the undifferentiated stigmatal ciliated cells. The boundary between them and the branchial cells, now "parietal cells," is easily recognisable owing to the presence on the apical plasmalemma of the latter of a thick glycocalyx (Fig. 20). Later, the stigma enlarges, arranges its cells in seven-row series flanked by parietal cells on both sides, and differentiates both apical cilia and microvilli. The main events of epithelial fusion and perforation occurring both in the blastozooid and the oozooid, are summarised in the diagram in Figure 21.

DISCUSSION

In this study, we have analysed the cellular mechanism of fusion and perforation of epithelia during the

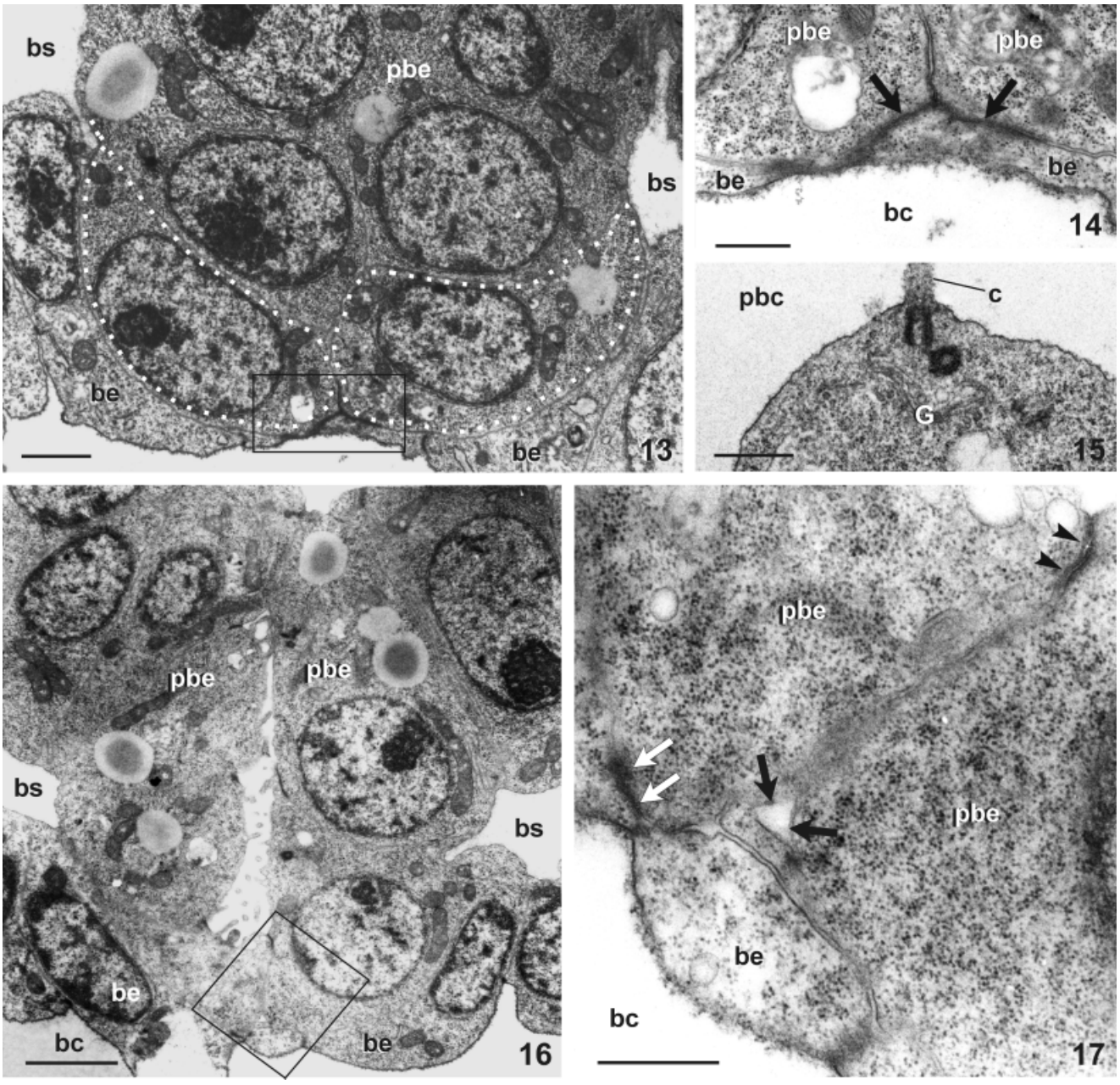
Fig. 9–12. Transmission electron microscopy of stigmatal primordia.

Fig. 9. A thickened area of peribranchial epithelium (pbe) extends toward the branchial epithelium (be); basal laminae (bl) separate the two epithelia. Inset: detail of the thick glycocalyx (arrowheads) covering branchial cells. bc, branchial chamber; bs, blood space; l, lipid droplet; n, nucleus; pbc, peribranchial chamber. Scale bar = 3 μm, 1.3 μm in inset.

Fig. 10. Tight junction between peribranchial cells (arrowheads). pbc, peribranchial chamber. Scale bar = 120 nm.

Fig. 11. Detail of basal plasmalemmata of peribranchial (pbe) and branchial (be) epithelial cells during adhesion. Note that the basal laminae (bl) are intermingled and compacted. bs, blood space; l, lipid droplet; m, mitochondrion; n, nucleus. Scale bar = 2 μm.

Fig. 12. Peribranchial (pbe) and branchial (be) epithelia are fused together. Note a long cell (marked by white dotted line) facing the peribranchial chamber (pbc) with the apical plasmalemma, strictly adhering to branchial cells, and showing the basal plasmalemma restricted to a small portion covered by the basal lamina (arrowheads). The boxed area is enlarged in Figure 15. bc, branchial chamber; bs, blood space; n, nucleus. Scale bar = 2.5 μm.



Figs. 13–17. Transmission electron microscopy of stigmatal primordia during epithelial perforation.

Figs. 13,14. Region of the primordium involved in epithelial fusion and perforation. The two cells bounded by the dotted white lines in Figure 13 belong to the peribranchial epithelium (pbe) and are undergoing change in polarity together with the adjacent branchial epithelium cells (be). Figure 14 is an enlargement of the area boxed in Figure 13. New presumptive tight junctions (arrows) are forming between peribranchial and branchial cells. The lateral membranes of branchial cells are reduced to areas of tight junctions. bc, branchial chamber; bs, blood space. Scale bars = 1.2 μm in Figure 13, 0.5 μm in Figure 14.

Fig. 15. Detail of boxed area in Figure 12 showing a Golgi complex (G) located in the apical area of a peribranchial cell and facing the peribranchial chamber (pbc). Basal body at base of cilium (c). Scale bar = 12 nm.

Fig. 16. A narrow canal of stigma primordium originates in peribranchial epithelium (pbe). At its bottom, the canal wall presses down on the branchial epithelium (be). Squared area is enlarged in Figure 17. bc, branchial chamber; bs, blood space. Scale bar = 2.2 μm.

Fig. 17. Detail of boxed area in Figure 16. The cell at the top belongs to the peribranchial epithelium (pbe) and is equipped with two sets of tight junctions: one (arrowheads) sealing it with the adjacent peribranchial cells, the other (white arrows) sealing it with the branchial epithelium (be). A similar pattern of junctions is exhibited by branchial cells involved in epithelial fusion. Note that a new cavity is forming between the peribranchial cells (black arrows). bc, branchial chamber. Scale bar = 0.6 μm.

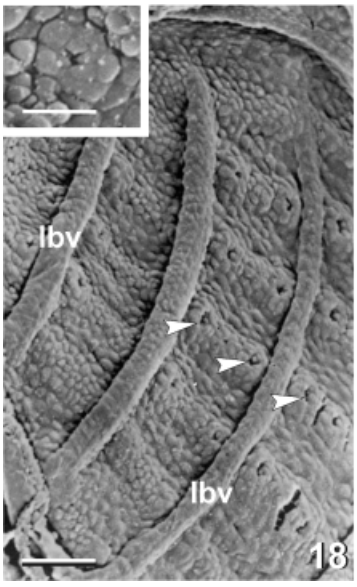
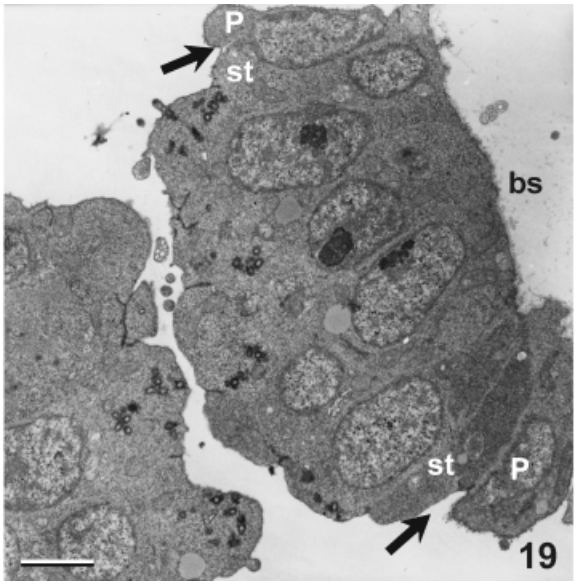
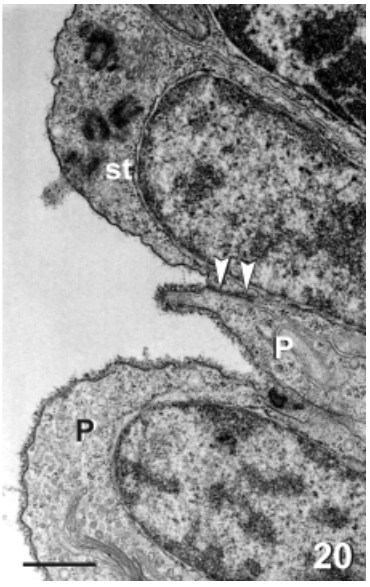


Fig. 18. Scanning electron photomicrograph of branchial wall showing early stigmatal opening (arrowheads). Inset: perforated stigma primordium. lbv, longitudinal branchial vessel. Scale bars = 23 μm, 7 μm in inset.





Figs. 19, 20. Early perforated but not yet ciliated stigma. Boundaries between stigmatal (st) and parietal (P) cells covered by dense glycocalyx is indicated by arrows in Figure 19 and enlarged in Figure 20. Arrowheads, newly formed tight junctions sealing branchial and peribranchial cells together. bs, blood space. Scale bars = 2.3 μm in Figure 19, 0.7 μm in Figure 20.

differentiation of the branchial fissures in the colonial ascidian *Botryllus schlosseri*. Both in the embryo and in the bud the process leads to the formation of fissures bordered by seven rows of ciliated cells, a characteristic organisation found typically in adult ascidians (Martinucci et al., 1988). This occurs during the formation of the specialised contact area between the branchial and peribranchial epithelia. Throughout the animal kingdom, adhesion and fusion of specific portions of epithelial surfaces is a frequent and extremely important process during embryonic development. Perhaps the best-known example of epithelial fusion is the closure of the neural tube by fusion of epithelial folds; however, although neurulation has often been investigated, the cellular basis driving the contact and fusion of the paired neural folds in the dorsal midline is poorly understood (Bard, 1990; Colas and Schoenwolf, 2001). Tissue fusion is usually referred to as "palate formation," in which the involved regions are disrupted and then withdraw, thus leaving two regions of naked mesenchyme which are then lost (Ferguson, 1988; Griffith and Hay, 1992). The case of branchial fissure differentiation in *B. schlosseri* is different from that of the palate and may be comparable to those situations in which epithelial integrity has to be maintained, such as neural fold fusion. Moreover, because in *B. schlosseri* no cells are eliminated by apoptosis, this process appears different from the ones observed in vertebrate development, in which the formation of openings occurs by means of programmed cell death (Sasaki et al., 1983; Watanabe et al., 1984; Nievelstein et al., 1998; Nishizaki et al., 1998; Qi et al., 2000; van der Werff et al., 2000).

The perforation of pharyngeal fissures during embryonic development represents a distinctive characteristic of all chordates, in which urochordates are considered close to the point of their origin. Thus, from an evolutionary point of view, they are of interest to understand the events that occurred at the transition from invertebrates to vertebrates. Moreover, ascidians, with their anatomic simplicity, their small genome and their wide diffusion, represent a suitable model for studying mechanisms of development, the genetic and cellular bases of which are in many cases shared with vertebrates (Wada et al. 1996, 1998; Shimeld, 1999; Shimeld and Holland, 2000; Manni et al., 2001). Apparently, the urochordate pharynx differs from that of vertebrates in that, in the former, the stigmata open into the peribranchial chambers and not directly to the exterior. Nevertheless, in both urochordates and vertebrates, the endoderm and the ectoderm participate in fissure formation. In *B. schlosseri* (Scott, 1934), as in other ascidians, the atrium derives from the invagination of the dorsoposterior ectoderm whose wall then adheres to the endodermal pharyngeal epithelium. Thus, this process is homologous to pharyngeal plate formation during vertebrate embryogenesis. However, it is noteworthy that, in *B. schlosseri*, two epithelia participate in the fusion without interposed mesenchymal tissue. This makes it simpler to follow the cellular dynamics in contrast with the situation in vertebrates.

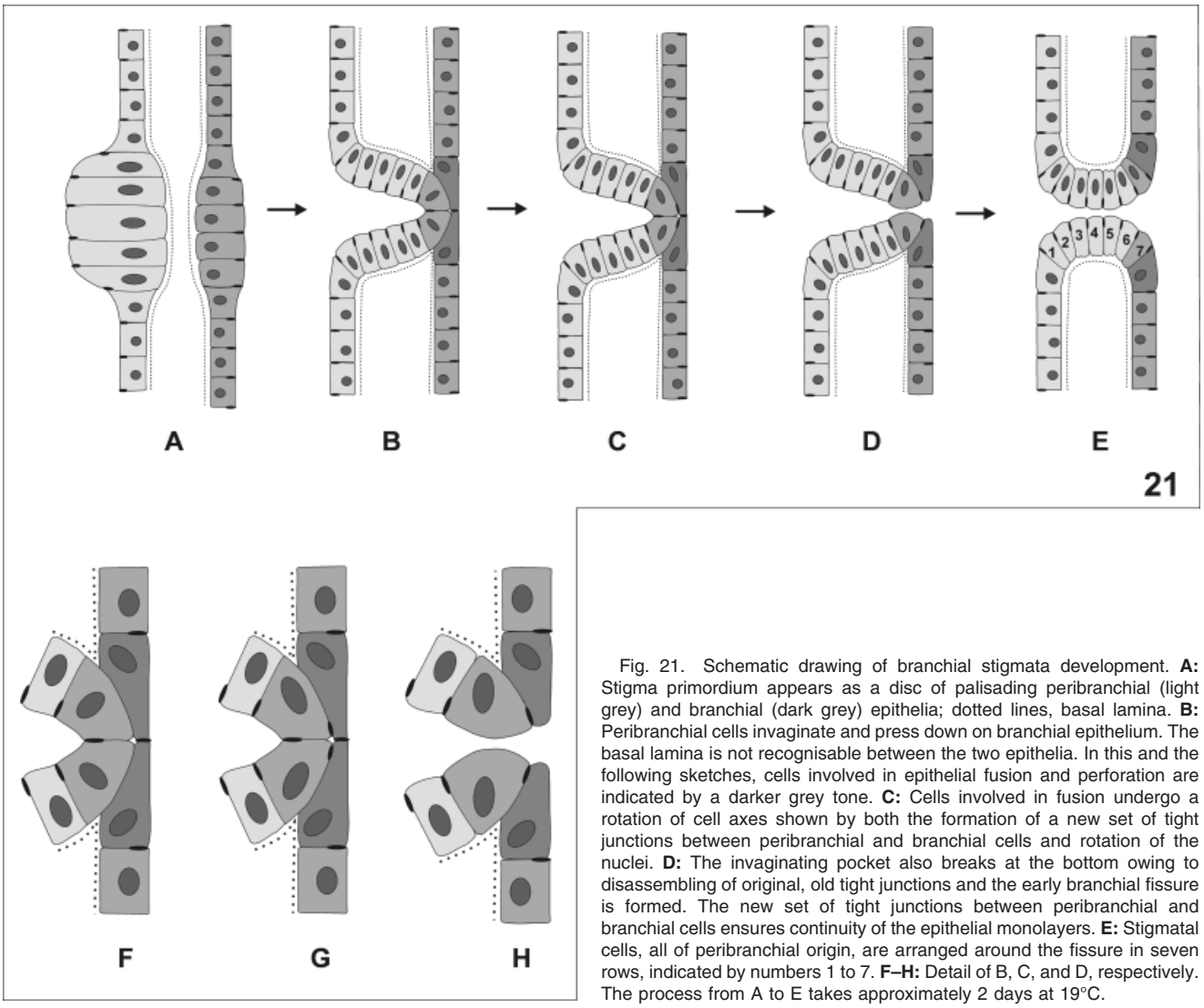


Fig. 21. Schematic drawing of branchial stigmata development. **A:** Stigma primordium appears as a disc of palisading peribranchial (light grey) and branchial (dark grey) epithelia; dotted lines, basal lamina. **B:** Peribranchial cells invaginate and press down on branchial epithelium. The basal lamina is not recognisable between the two epithelia. In this and the following sketches, cells involved in epithelial fusion and perforation are indicated by a darker grey tone. **C:** Cells involved in fusion undergo a rotation of cell axes shown by both the formation of a new set of tight junctions between peribranchial and branchial cells and rotation of the nuclei. **D:** The invaginating pocket also breaks at the bottom owing to disassembling of original, old tight junctions and the early branchial fissure is formed. The new set of tight junctions between peribranchial and branchial cells ensures continuity of the epithelial monolayers. **E:** Stigmatal cells, all of peribranchial origin, are arranged around the fissure in seven rows, indicated by numbers 1 to 7. **F–H:** Detail of B, C, and D, respectively. The process from A to E takes approximately 2 days at 19°C.

It is to be noted that, in all ascidians, the final organisation of the stigmata is rows of seven ciliated cells. This pattern was previously reported for all the adult ascidians examined, both mature solitary individuals and blastozooids in colonial species, independent of the elliptical of spiral shape of the fissures (Martinucci et al., 1987; Burighel and Cloney, 1997). In *B. schlosseri*, we found that both the organisation of stigmatal rings with rows of seven ciliated cells and the mechanism of fissure formation from the peribranchial and branchial epithelium is the same both in oozooid and blastozooid development. The presence of common developmental mechanisms in embryo and bud were also previously reported during neurogenesis of *B. schlosseri* (Burighel et al., 1998; Manni et al., 1999).

Cell Reorganisation in Epithelial Fusion and Perforation During Stigma Development

In *B. schlosseri*, the first sign of morphogenetic activity of the peribranchial epithelium is the appearance of small round domains, or buttons, in which cells palisade and elongate toward the branchial epithelium. Cells of the opposing epithelia approach each other, the intervening basal lamina intermingles and is successively degraded, and new cell–cell contacts are established between adhering cells. Thus, the process of epithelial fusion involves the apposition of part of the opposing basal membranes; no infiltration of cells from one layer into the opposite was recognised. In contrast, in the perforation of the chick closing plates and oral membrane, hamster oral membrane, and chick foramina secunda, cells of opposed epithelia interdigitate to create a monolayered epithelium which rapidly thins and breaks (Waterman, 1977, 1985; Morse and Hendrix, 1980; Waterman and Schoenwolf, 1980).

It is noteworthy that the extracellular matrix must be an important source of signals, which can be transduced into the nucleus to give specific gene expression (Rodriguez-Boulan and Nelson, 1989; Clark and

Brugge, 1995), and it must be supposed that peribranchial and branchial cells, when coming into contact by means of their own basal membrane, are subject to new information inducing morphogenetic activity. Our data suggest that this represents a critical event in triggering the successive changes in polarity and remodelling of cells. The controlled degradation of the extracellular matrix is an essential part of normal development and is accomplished by a set of enzymes collectively called matrix-degrading metalloproteinases (Matrisian, 1992). This controlled event precedes the peribranchial and branchial epithelial fusion, as seen during the perforation of the chick closing plates (Waterman, 1985).

When the basal lamina disappears, cell–cell contacts are established between the peribranchial and branchial cells, as indicated by the fact that the intercellular space separating plasma membranes is reduced to a 10- to 12-nm cleft, like that separating the cells of the same epithelium. The new cell contact may trigger the change in cell polarity which characterizes cell rearrangement. That cell–cell interactions play a role in the development and maintenance of epithelial cell polarity is clearly demonstrated by in vitro experiments performed on Madin-Darby canine kidney (MDCK) cells (Vega-Salas et al., 1987; Wang et al., 1990a): cell–cell contact is sufficient to trigger the segregation of marker proteins of the apical and basal-lateral membrane domains to distinct regions of the membrane. There are also various examples of the ability of epithelial cells to regenerate or change their surface polarity (Rodriguez-Boulan and Nelson, 1989) in vitro, in passage from a round cell suspension to an asymmetric monolayer (Cereijido et al., 1980) and, in vivo, in the generation of tubule cells from mesenchymal cells during kidney development (Davies, 1996; Horster et al., 1997).

It is interesting to note that in *B. schlosseri* cells involved in fusion undergo a movement of their epithelial axis: the original basal-apical axis shifts toward a basal-lateral axis, whereas the original lateral membrane acquires the features of the apical side. The change in cell axis is not a reversal, as demonstrated by the fact that the part of basal plasmalemma of cells involved in fusion becomes lateral, whereas the remaining portion, although small, never looses its contact with the basal lamina. The rotation of approximately 90 degrees of the cell axis involves both the peribranchial and branchial cells, although it is of note that the direction of rotation is the opposite.

The shifting of cell axis is indicated precociously by a change in the orientation of the nucleus and Golgi complex relative to the location of the new apical side. This change is clearly evident in peribranchial cells, which are columnar, compared with the branchial cells, which are highly attenuated. Changes in the distribution of the nucleus and Golgi complex were the first indications of the reversal of cell polarity in MDCK cells too, upon transfer of epithelia cysts formed in suspension culture to a collagen gel (Wang et al., 1990b).

The presence of tight junctions is a cellular property shared by urochordates and vertebrates among the Deuterostomes (Lane et al., 1994). In ascidians, they are well developed in the branchial basket and other tissues (Lane et al., 1986; Martinucci et al., 1988). Our observations reveal that a crucial step in forming the branchial fissure is the appearance of a new tight junction, so that just before the stigma opens, cells are equipped with two sets of zonulae occludentes. Their presence indicates that a change in cell polarity has occurred (Cereijido et al., 2000). In general, tight junctions, located at the boundary between basolateral and apical regions, in a cell layer, produce a selective barrier to the diffusion of ions and macromolecules. They also form a boundary within the plasma membrane itself (fence function) separating apical and basolateral domains and restricting the lateral mobility of membrane components (Tsukita et al., 2001). Their formation has been seen to be critical for embryonic patterning and organisation (Fesenko et al., 2000; Fleming et al., 2000). Our observations in *B. schlosseri* are in agreement with recent data showing that tight junctional biogenesis occurs after the acquisition of cell polarity, because the new junctions appear in stigma primordium, when the cells begin to exhibit a new polarity. The temporary persistence of two tight junctional domains in the same cell has been previously described in other systems, such as the reversal of cell polarity in MDCK cells (Wang et al., 1990b). In vivo, similar situations were reported during the rupture of hamster and chick oral membrane, and in the formation of semicircular canals in the zebrafish; in these cases, however, tight junctions were not unequivocally identified (Waterman, 1977; Waterman and Schoenwolf, 1980; Waterman and Bell, 1984).

Disassembling of the original, old tight junctions seems to be the final step in branchial fissure opening of *B. schlosseri*. However, the maintaining of epithelial integrity and the subsequent separation of the blood from the luminal compartment of the chambers would be ensured by the new tight junctions just formed. The dynamic and plastic behaviour of tight junctions have often been reported. It is well known that neutrophils regularly migrate across mucosal epithelia and early germ cells pass through the tight junctions of the Sertoli cells to reach the adlumenal compartment of the testis (Byers and Pelletier, 1992). *In vitro*, tight junctional disassembling has been precisely documented (Wang et al., 1990b). Thus a sequential order of assembling and disassembling tight junctions would ensure in *B. schlosseri* the epithelial integrity for maintenance of the blood compartment and the realisation of the branchial fissures. With regard to several of the above documented aspects (homology, presence of simple epithelia, phylogenetic significance, etc.), *B. schlosseri* represents an interesting model for further investiga-

tion of the cellular mechanisms involved in fusion and perforation of epithelia.

EXPERIMENTAL PROCEDURES

Materials

The ascidian *Botryllus schlosseri* (family Styelidae, order Stolidobranchia) forms colonies composed of a large number of small zooids (blastozooids), embedded in a common tunic and arranged in star-shaped systems. Animals used in this study were collected in the lagoon of Venice and cultured on glass in the laboratory (at 18°C) following Sabbadin's (1955) technique.

The transparency of the colonies allowed us to follow the daily development in vivo of embryos and buds under the stereomicroscope, thereby permitting the selection of appropriate stages. The developmental stages of both embryos and buds are synchronous and closely correlated with those of the parent. Three blastogenetic generations coexist in the colony: the filtering adults, their buds, and the buds that the buds themselves produce. Weekly, at approximately 18°C, all adults regress and are reabsorbed; their buds become the new adults and begin filtering. Embryos are retained by the parent and develop after approximately 1 week of gestation. Larvae hatch just before the adults regress and, after a short period of swimming life, undergo metamorphosis, giving rise to the sessile oozooids, which reproduce asexually by budding. After some blastogenetic generations, blastozooids reach maturity and can reproduce sexually, thereby producing new larvae. Blastozooids are similar in structure to the filtering oozooid, but the latter is easily recognisable by the presence of four to five protostigmata oriented dorsoventrally, whereas blastozooids possess parallel rows of small elliptical stigmata oriented anteroposteriorly.

Embryos and buds were removed from the dissected parents by using a thin tungsten needle, whereas larvae at various stages of metamorphosis were pipetted directly from the holding tanks into the fixative liquid. The developmental stages of embryos were based on correlation between the developmental stage of the colony and embryogenesis and the gross anatomic larval features; buds were collected following Sabbadin's (1955) stages of development, which consider this feature: appearance of the bud primordium in form of a thickening of the atrial epithelium, which folds to close into a vesicle surrounded by the epidermis. This vesicle begins to deform to give the main primordia of the branchial and peribranchial chambers and gut and the bud producing the primordia of the daughter; its heart then begins beating, while the newly formed buds develop.

Transmission Electron Microscopy

Embryos 3 days, 2 days, and 1 day before hatching, swimming larvae, and buds were fixed in 1.5% glutaraldehyde buffered with 0.2 M sodium cacodylate, pH 7.4, plus 1.6% NaCl. After washing in buffer and postfixation in 1% OsO_4 in 0.2 M cacodylate buffer plus 1.6% NaCl, the specimens were dehydrated and embedded in Araldite. Thick sections (1 μm) were counterstained with toluidine blue; thin sections were sectioned and given contrast by staining with uranyl acetate and lead citrate. Photomicrographs were taken with a Hitachi H-600 electron microscope operated at 80 kV.

Scanning Electron Microscopy

Zooids were fixed as described for transmission electron microscopy. After dehydration, zooids were dissected to expose the pharynx. Specimens were then critical-point dried, sputter-coated with gold palladium, and observed by using a Cambridge Stereoscan 260.

Photography

All figures were acquired with a Duoscan (Agfa), had levels adjusted, and were collated and typeset in Corel Draw 9.

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