Membrane fusion as a morphogenetic force in nematode development

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Summary – In *Caenorhabditis elegans* almost all the epithelial cells fuse to form permanent syncytia. Cells in the vulva and hypodermis fuse autonomously to produce ring shaped cells with defined structures and functions. Analysis of temporal and spatial sequence of events together with ultrastructural characterisation of cell fusion intermediates show that fusion pores in specific domains of the membranes dilate and subsequently vesicles are formed. The fusomorphogenetic hypothesis states that these vesicles are targeted to different domains of the plasma membrane where they fuse, thereby causing changes in cell shape. It is proposed that cell fusion and polarised membrane recycling are involved in the formation of ring cells. Fusomorphogenesis is a working model to investigate the forces that drive pattern formation and generate diversity of developmental mechanisms in nematodes.

Résumé – ***La fusion membranaire comme force morphogénétique au cours du développement des nématodes –*** Chez *Caenorhabditis elegans*, presque toutes les cellules épithéliales fusionnent pour former des syncytia permanents. Les cellules de la vulve et de l'hypoderme fusionnent de façon autonome et produisent des cellules en forme d'anneau avec des structures et des fonctions définies. L'analyse de la séquence temporelle et spatiale des événements, alliée à la caractérisation ultrastructurale des intermédiaires de fusion cellulaire, montre que des pores de fusion se dilatent dans des domaines membranaires spécifiques et que des vésicules sont par la suite formées. L'hypothèse fusomorphogénétique suggère que ces vésicules sont ciblées vers des domaines différents de la membrane plasmique, où elles fusionnent, provoquant ainsi des changements de forme cellulaire. Il est proposé que la fusion cellulaire et le recyclage polarisé de membranes soient considérés comme impliqués dans la formation des cellules en anneau. La fusomorphogénèse est un guide de travail pour étudier les forces qui entraînent la formation d'un modèle spatial et engendrent la diversité des mécanismes de développement chez les nématodes.

Keywords – *Caenorhabditis elegans*, cell fusion, cell migration, hypodermis, invagination, membrane recycling, Nematoda, organogenesis, pattern formation, polarised secretion, syncytia, vulva.

Cell-cell fusion is a widespread process required for fertilisation and conjugation between gametes and in the formation of many somatic tissues (Yanagimachi, 1988; Hernandez *et al.*, 1996). Multinucleated or syncytial cells can be generated by cell fusion, by cell division cycles without cytokinesis, or by a combination of both of these processes. Many different developmental processes involve syncytia formation. These syncytia can be of two types: transient and permanent.

Transient syncytial cells have been found in the gonads of plants, nematodes and mammals where they participate in gametogenesis. In the early embryonic development of many arthropods the zygote undergoes nuclear proliferation without cytokinesis forming a transient syncytial cell that eventually cellularises to give rise to all the embryonic cells (*e.g.*, syncytial blastoderm in *Drosophila*; Gilbert, 1997; Wolpert *et al.*, 1998). In humans, large binucleate megakaryoblasts give rise to polyploid megakaryocytes that shed platelets (Wheater *et al.*, 1979). In annelid embryos (*e.g.*, leech) transient syncytial yolk cells are formed by stereotyped non-autonomous cell fusion between cells derived from endodermal, mesodermal and ectodermal lineages. These syncytial yolk cells eventually cellularise to form part of the intestine (Isaksen *et al.*, 1999). In *Caenorhabditis elegans* a pair of tail spike cells fuse together, form a bundle of filaments in the tip of the tail, and then die several hours after birth (Sulston *et al.*, 1983). Thus, the formation of transient syncytia can be accomplished by three different strategies. First, by the process of cell division cycle without cytokinesis during

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gametogenesis (*e.g.*, in humans and nematodes) and during the syncytial blastoderm stage of *Drosophila* embryos. Second, by inductive cell fusion in leech embryos, and third, by cell fusion followed by programmed cell death in the tail-spike of *C. elegans* embryos. In summary, transient syncytia formation allows an efficient way to regulate the generation of a mass of cellular components followed by the process of cellularisation or programmed cell death.

Permanent syncytial cells can be the final result in a developmental process, which means that the ultimate fate of a certain cell is to fuse with another mononucleated cell or to a pre-existing multinucleated cell (Podbilewicz & White, 1994). Formation of permanent syncytial cells has been found in many dynamic developmental processes. Myoblasts fuse during muscle development (Knudsen & Horwitz, 1977; Wakelam, 1988; Blau *et al.*, 1993; Rushton *et al.*, 1995; Doberstein *et al.*, 1997). Monocytes fuse to form osteoclasts involved in bone formation and resorption (Jee & Nolan, 1963; Baron *et al.*, 1986; Takahashi *et al.*, 1994; Filvaroff & Derynck, 1998). Cytotrophoblasts fuse to form syncytial trophoblasts during placental development (Cross *et al.*, 1994; West *et al.*, 1995). Mesenchymal cells fuse to form larval skeleton in sea urchin (Hodor & Ettensohn, 1998), and one third of all the somatic cells generated during *Caenorhabditis elegans* development fuse to form syncytial cells (Podbilewicz & White, 1994; Podbilewicz, 1996).

Cell fusion in *C. elegans*

Cell fusion is the most common cell fate in *C. elegans.* In hermaphrodites, 298 different somatic cells, accounting for 31% of the somatic nuclei, fuse in specific spatial and temporal patterns to form 43 syncytial cells (Table 1). On top of these somatic cell fusions, practically all the 300 sperm produced during the life of a single virgin hermaphrodite fuse with oocytes as they enter the spermatheca and around 1400 oocytes fuse with male derived sperm when there is cross-fertilisation (Kimble & Ward, 1988). Several hermaphrodites, mated with a single male, are capable of producing more than 2500 cross progeny (Hodgkin, 1983). Several mutations affecting sperm-egg fusion have been identified in *C. elegans* and a sperm membrane protein required for fertilisation has been recently characterised (Singson *et al.*, 1998).

Most somatic cell fusions occur between epithelial cells in the hypodermis, excretory gland cells, vulva, uterus and pharynx. Some pharyngeal muscle cells also fuse (Table 1). Cell fusions are specific and generate an invariant pattern of multinucleated cells (Albertson & Thomson, 1976; Sulston & Horvitz, 1977; Sulston *et al.*, 1983; Kenyon, 1986; Baird *et al.*, 1991; Podbilewicz & White, 1994; Newman *et al.*, 1996; Nguyen *et al.*, 1999; Sharma-Kishore *et al.*, 1999). The process of cell fusion appears to be cell autonomous in *C. elegans* and independent of the founder cell that originated the fusing cells based on the following independent evidence.

First, fusion occurs between descendants of four different founder cells and with the exception of the founder cells E and D all the other founder cells (AB, C, MS, P4) have descendants that fuse (Sulston & Horvitz, 1977; Sulston *et al.*, 1983; Podbilewicz & White, 1994). Second, patterning of the anteroposterior body axis by combinatorial expression of genes of the *C. elegans* Hox cluster (homeotic selector genes) in the ventral ectodermal Pn.p cells regulate their fusion autonomously (Salser *et al.*, 1993; Wang *et al.*, 1993; Cowing & Kenyon, 1996; Ch'ng & Kenyon, 1999). Third, *lin-15* expression in a pre-existing hyp7 syncytium, that is one of the fusion partners, is required for the fusion of certain uninduced vulva precursor cells (VPCs) to the hyp7 syncytium itself (Herman & Hedgecock, 1990). Fourth, laser ablation of one of the two cells that fuse in the vulva results in self-fusion of the surviving cell (Sharma-Kishore *et al.*, 1999). In other words, separate domains of a single fusion-competent cell can fuse to one another in the absence of the natural fusion partner. Fifth, incomplete 'real' and pseudovulvae in a *let-60/ras* gain-of-function mutation form complete rings that undergo self-fusion autonomously (Shemer *et al.*, 2000). In summary, specific spatial and temporal patterns of numerous cell fusion events are regulated autonomously in the fusion-competent cells. Inductive regulation of cell fusion by cells that do not themselves play a role in the fusion process has not been found in *C. elegans.*

This review will analyse the role of cell fusion in the formation of the hypodermis during embryonic morphogenesis and the involvement of cell fusion in the invagination of the vulva during postembryonic development in *C. elegans.* The formation of these organs involves stereotyped rearrangements of small cell populations and the generation of permanent syncytial ring cells with very specific and invariant morphologies and functions. Comparative analyses of the role of cell fusion during the formation of the hypodermis and the vulva between *C. elegans* and other nematodes will allow a better understanding of the generation of diversity and the evolution of developmental mechanisms.

Table 1. *Summary of all the reported cell fusions in the* Caenorhabditis elegans *hermaphrodite according to the system, organ, tissue, cell and number of nuclei per syncytial cell.*

System	Organ	Tissue	Cell	Nuclei
Gastro-intestinal	pharynx[1,2,3]	pharyngeal muscles	m1	6
			m2 (3X2)	6
			m3 (3X2)	6
			m4 (3X2)	6
			m5 (3X2)	6
		epithelial marginal cell	mc3	3
		gland cell in pharynx	g1	3
Excretory	excretory glands[1,3]	epithelial	exc. gl.	2
Skin	hypodermis[1,3,4]	epithelial	hyp1	3
			hyp2	2
			hyp3	2
			hyp4	3
			hyp5	2
			hyp6+hyp7[5]	139
			hyp10	2
			seam (l)	15
			seam (r)	15
Reproductive	vulva[4,6]	epithelial	vulA	4
			vulC	4
			vulD	2
			vulE	4
			vulF	4
	uterus[7]	epithelial	utse+AC	9
			ut1 (2X4)	8
			ut2 (2X4)	8
			ut3 (2X4)	8
			ut4 (2X6)	12
			sujn (2X4)	8
			plug	2
			du	4
4 systems	5 organs	2 tissue types	43 cells	298 nuclei

[1](Sulston & Horvitz, 1977); [2](Albertson & Thomson, 1976); [3](White, 1988); [4](Podbilewicz & White, 1994); [5](Yochem *et al.*, 1998); [6](Sharma-Kishore *et al.*, 1999); [7](Newman *et al.*, 1996).

Hypodermal rings and cell fusions during morphogenesis

To study epithelial morphogenesis and cell fusion in developing *C. elegans* it is necessary to follow the membranes of cells before, during and after the fusion events. Since the cell boundaries are not easily resolved using Nomarski differential interference contrast microscopy the standard procedure in nematodes is to stain the worms and use immunocytochemistry, immunofluorescence or electron microscopy (White *et al.*, 1976, 1986; White, 1988; Podbilewicz & White, 1994; Podbilewicz, 1996; Sharma-Kishore *et al.*, 1999). The monoclonal antibody MH27 that recognises a component of the adherens junctions has been widely used to follow the apical junctions between polarised cells in *C. elegans* and other nematodes (Kenyon, 1986; Priess & Hirsh, 1986; Baird *et al.*, 1991; Francis & Waterston, 1991; Austin & Kenyon, 1994; Podbilewicz & White, 1994; Hall, 1996; Newman *et al.*, 1996; Podbilewicz, 1996; Fitch, 1997; Nguyen *et al.*, 1999; Sharma-Kishore *et al.*, 1999). More recently the gene encoding the MH27 antigen was cloned and a reporter fusion protein combining the MH27 protein with green fluores-

cent protein (MH27-GFP) has been used in living worms (Mohler *et al.*, 1998).

Developmental cell fusion in *C. elegans* is a polarised event during pattern formation just as the orientation of the cleavage during cytokinesis is critical to determine asymmetries like the body axes or the position of a certain organ. Most cell fusions, as occur with most cell divisions in *C. elegans*, are invariantly longitudinal or anterior-posterior, some cell fusions are transverse or left-right and very few cell fusions are dorso-ventral (Podbilewicz & White, 1994). Cell fusion can be homotypic between like cells or heterotypic between cells of different origins, shapes and functions (Podbilewicz & White, 1994; Sharma-Kishore *et al.*, 1999). Finally, as occurs in vertebrate muscles, epithelial mononucleated cells in *C. elegans* can fuse with other mononucleated cells, with different syncytial cells and, in some mutants, two distinct membrane domains of a single mononucleated cell may fuse generating self fusion (Podbilewicz & White, 1994; Sharma-Kishore *et al.*, 1999; Shemer *et al.*, 2000). In a few cases, reproducible fusion between existing syncytia occurs invariantly both in the hypodermis (Yochem *et al.*, 1998) and the vulva (Sharma-Kishore *et al.*, 1999). The great diversity in the topology of cell fusion may explain elongation, the generation of different shapes and in particular the formation of stable rings in the hypodermis and the vulva.

Hypodermal cell fusions during embryonic development

By following the adherens junctions of developing worms in stereo-3-D and stereo-4-D reconstructions of confocal images of nematodes, it was found that the first stereotyped cell fusions after sperm-egg fusion occur after the embryo is completely enclosed by an epithelial monolayer composed of five rows of hypodermal cells (Podbilewicz & White, 1994; Mohler & White, 1998) (Fig. 1). These five rows of epithelial cells enclosing the embryo originate from six rows of cells that were born between 200 and 250 min after first cleavage (Sulston *et al.*, 1983; Podbilewicz & White, 1994). At 250 min after first cleavage, the six rows can be visualised at the dorsal hypodermis using MH27 antibody (Podbilewicz & White, 1994). Between 250 and 390 min the middle two rows rearrange by interdigitation to form a single row of dorsal cells, flanked by two rows of lateral seam cells (Sulston *et al.*, 1983; Podbilewicz & White, 1994; Williams-Masson *et al.*, 1998). While the dorsal cells migrate by forming and breaking adherens junctions between them, the most external cells on the left and right side migrate towards the ventral side of the embryo (Podbilewicz & White, 1994; Williams-Masson *et al.*, 1997). They encounter each other and become attached by forming new adherens junctions (Podbilewicz & White, 1994; Williams-Masson *et al.*, 1997). Thus, simultaneous interdigitations in the dorsal hypodermis and migrations towards the ventral side completely enclose the embryo before cell fusion and elongation of the embryo (morphogenesis).

Ventral transverse left-right cell fusions $\mathbf{F_T}$

The first cell fusion in the developing embryo occurs between two cells in the anterior ventral hypodermis just before the onset of elongation (Podbilewicz & White, 1994). The cells establish new attachments that are aligned longitudinally and then the membranes fuse as revealed by their disappearance using MH27 antibody staining, membrane vital dye, MH27-GFP reporter and electron microscopy (Podbilewicz & White, 1994; Mohler *et al.*, 1998). Many, but not all, epithelial cell fusions start around 3 h after the fusing partners are born (Hedgecock & Thomson, 1982; Hedgecock & White, 1985; Podbilewicz & White, 1994). Thus, the first somatic cell fusion in the hypodermis appears to be triggered by the enclosure of the embryo by a pair of left-right ventral cells around 340 min after first cleavage (Podbilewicz & White, 1994). These 'leading cells' not only appear to initiate ventral enclosure (Williams-Masson *et al.*, 1997), they are also leading cells in the fusion process (Podbilewicz & White, 1994). This pioneer transverse cell fusion ($\mathbf{F_T}$) in the hypodermis is the first of a series of stereotyped fusions. The order of cell fusion events is not completely invariant but follows a certain anterior to posterior pattern (Podbilewicz & White, 1994). Ten independent pairs of left-right ventral cells fuse during embryogenesis and are instrumental in the formation of the eleven cells hyp1-11 that form eleven concentric rings that enclose the elongating embryo (Sulston *et al.*, 1983; Podbilewicz & White, 1994).

Dorsal longitudinal anterior-posterior cell fusions $\mathbf{F_L}$

While during embryogenesis all ventral fusions are pairwise transverse fusions ($\mathbf{F_T}$), all the dorsal cell fusions have an anterior-posterior longitudinal orientation ($\mathbf{F_L}$) (Podbilewicz & White, 1994). Fig. 1 shows dorsal views of an embryo expressing MH27-GFP in the adherens junctions of all epithelial cells and revealing the

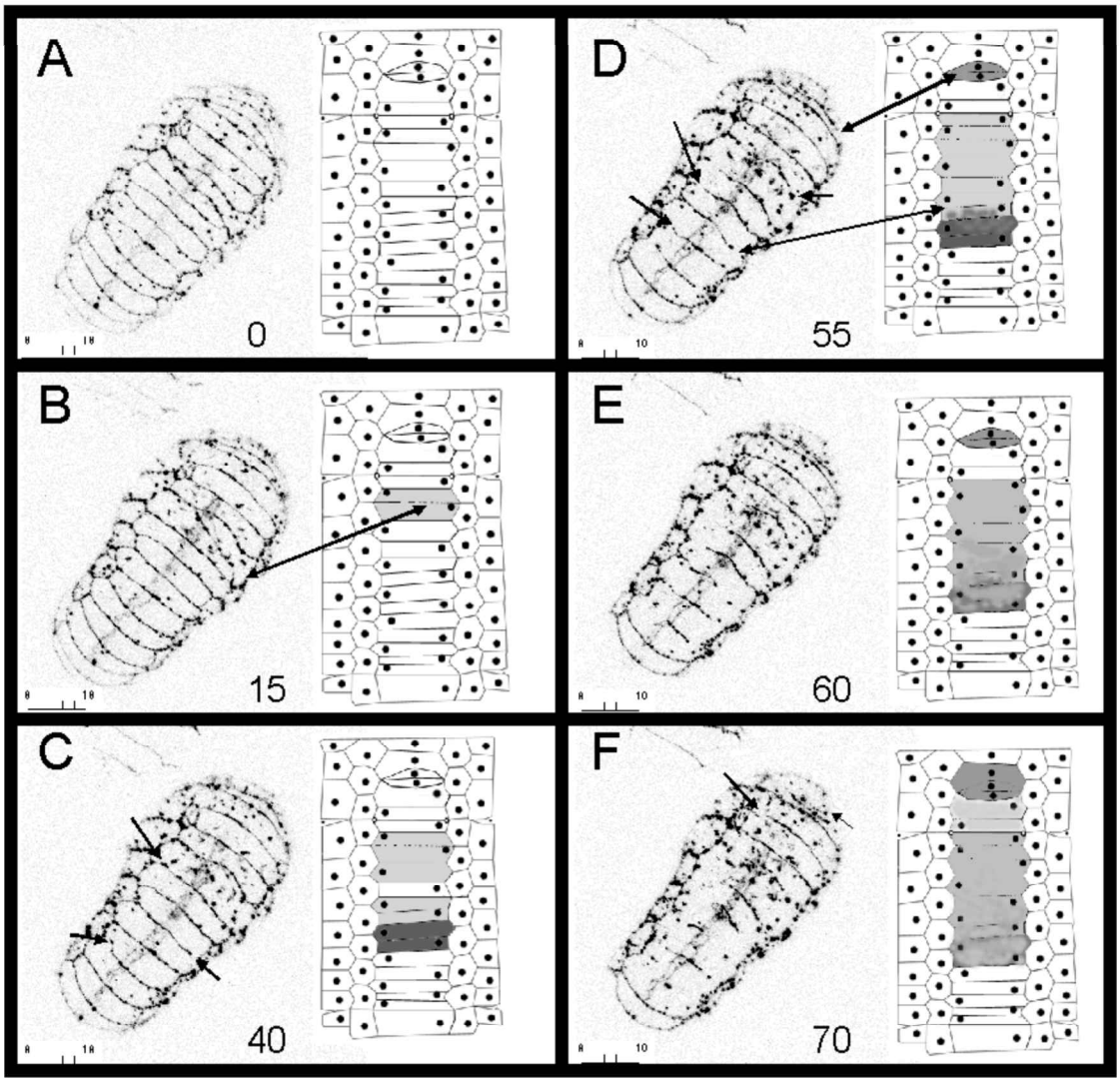

Fig. 1. *Hypodermal dorsal fusions during morphogenesis of the embryo. The images on the left side of each panel are projections of a living embryo expressing MH27-GFP on the adherens junctions and revealing the apical boundaries between epithelial cells. The diagrams are cylindrical projections of the embryo cut open along the ventral midline and viewed from outside showing the position of 65 hypodermal cells organised in five rows. Not all the cells in the diagram are seen in the images. Black dots are nuclei and cell fusion is represented by a discontinuity of the membranes and the presence of a gray tone in the cytoplasm of the fusing cells. The fusions in the embryo occur during elongation. A: The starting time* (t = *0) is the comma stage, before elongation; B: Intermediate showing the first dorsal fusion between cells that will be part of the hyp7 syncytium; C: Top arrow points to the fusion between the anterior binucleate cell and an adjacent posterior cell; the two other arrows show the initial fusion between two pairs of cells; D: The anterior arrow shows the first dorsal fusion in hyp6 syncytium; four additional cell fusion events form a dorsal syncytium containing nine nuclei; E: The boundaries between cells continue to disappear at a rate of 202 ± 46 nm/min and cytoplasm mixing is shown in the diagram; F: One cell fuses to hyp6 (top arrow) and two cells fuse to form a binucleate cell that will eventually join the hyp7 syncytium; at the end of embryogenesis hyp6 syncytial ring will contain four dorsal and two ventral nuclei while hyp7 will contain 17 dorsal nuclei and six ventral nuclei. (The images are from time-lapse 4D-confocal microscopic reconstruction of MH27-GFP expressing embryos; the numbers in the bottom of each panel refer to time points in minutes; arrows point to the initial discontinuity of the GFP staining that reveals the fusion between membranes; in B and D the position of the boundaries that fuse are shown in the diagrams with double headed arrows for three cell fusions; anterior is towards the top of the page and the images are dorsal views; scale bars = 10 μm.)*

apical boundaries. On the right side of each panel is the corresponding schematic representation of a cylindrical projection of all the major hypodermal cells making up the central body region of the embryo cut open along the ventral midline and viewed from the outside (Podbilewicz & White, 1994). These time points from a time lapse movie show the dynamics of eleven $\mathbf{F_L}$ events. The rate of disappearance of the membranes and adherens junctions between fusing cells is around 200 nm/min at 17.5°C (Podbilewicz, unpubl.). This means that the disappearance of longitudinal membranes between two dorsal epithelial cells that cover approximately 20 μm take 100 min at 17.5°C when there is a single fusion origin. However, multiple foci of membrane fusion have been observed between pairs of fusing cells resulting in a shorter time for completion of the fusion process (Fig. 1). The rate of disappearance of FM 4-64, a fluorescent membrane probe (Mohler *et al.*, 1998), and the rate of disappearance of a marker of adherens junctions (MH27-GFP) were around 400 nm/min at 21°C (Podbilewicz, unpubl.). In summary, the rate of $\mathbf{F_L}$ cell fusion can be reproducibly measured in living embryos.

Dorso-ventral fusions $\mathbf{F_{DV}}$

Serial section electron microscopy has shown that the dorsal syncytial cells fuse with specific ventral binucleate syncytia during embryogenesis to form eight syncytial rings that surround the embryo (Podbilewicz & White, 1994). These dorso-ventral fusions ($\mathbf{F_{DV}}$) occur under the lateral seam cells and do not form stable apical adherens junctions before the fusions occur. Transient micro-adherens junctions between filipodia may form during $\mathbf{F_{DV}}$, but they have not been detected probably due to their small size and short life time. At the end of embryogenesis the hypodermal cells of the head form six concentric rings (hyp1-hyp6) containing respectively 3, 2, 2, 3, 2, and 6 nuclei (Sulston *et al.*, 1983; White, 1988; Podbilewicz & White, 1994). The embryonic hyp7 syncytium contains 23 nuclei and is cylindrical in its anterior and posterior part but not in the midbody (Sulston *et al.*, 1983; White, 1988; Podbilewicz & White, 1994). During postembryonic development additional 133 cells invariantly fuse to hyp7 resulting in a complete ring cell that is pierced at various specific locations by the vulva, anus, excretory pore and sensory endings. In the tail, posterior to hyp7, there are four cells (hyp8-hyp11) of which only hyp10 is binucleate (Sulston *et al.*, 1983; White, 1988; Podbilewicz & White, 1994; Nguyen *et al.*, 1999). During postembryonic development hyp6 and hyp7 undergo a $\mathbf{F_L}$ event forming the largest syncytium in the adult (Yochem *et al.*, 1998; Table 1). Of the 186 hypodermal nuclei contained in the adult hypodermis only three are not part of syncytial rings formed by cell fusion. The formation of ring cells linked by cylindrical adherens junctions is a common process in *C. elegans* development. In the next section the formation of the vulva that also involves migrations, ordered fusions and formation of ring cells will be analysed.

Formation of vulval rings and cell fusions drive invagination

Invagination from the Latin *invaginare* means to sheathe or the action of sheathing or inverting (Little *et al.*, 1973). Vagina (sheath) is the membranous canal leading from the vulva to the uterus in female mammals and a genital passage in other animals. Vulva is the external reproductive organ or the opening of that organ (Little *et al.*, 1973). In the *C. elegans* hermaphrodite the genes that specify the cell fates in the vulva are known in great detail (for reviews, see Greenwald, 1997; Kornfeld, 1997) and the diversity of the inductive interactions responsible for the induction of the vulva in different nematodes is known in some detail (Félix & Sternberg, 1997; Sommer, 1997). Recently the complete morphogenetic cellular events that form the vulva have been characterised (Sharma-Kishore *et al.*, 1999). However, very little is known about the molecular mechanisms and the evolution of the invagination of the vulva in nematodes. It is generally accepted that changes in cell shapes and cell movements generate physical forces responsible for pattern formation (Trinkaus, 1984; Wolpert *et al.*, 1998). In particular, the formation of a tube from a sheath of cells is accomplished during gastrulation, neurulation and organ formation (*e.g.*, hair folicules and kidney) through localised constriction of apical domains of a few cells in a sheet of cells and/or cell migration (Schoenwolf, 1984; Kimberly & Hardin, 1998; Lawson & England, 1998). This polarised change in cell shape and cell migrations can cause the folding, invagination and eventually the formation of a tube (Papan & Campos-Ortega, 1994; Gilbert, 1997; Davidson & Keller, 1999). The formation of the vulva in *C. elegans* is accomplished by changes in cellular contacts and the formation of a stack of seven cellular rings (Sharma-Kishore *et al.*, 1999). To my knowledge the cellular strategy used in vulva formation has not been described in other biological invaginations and may or may not be conserved among nematodes.

PHASES OF VULVA FORMATION IN *C.ELEGANS*

Vulva formation can be divided into three phases.

Short-range cell migration and ring stacking

A symmetrical and palindromic sheet of 22 vulval epithelial cells is generated in the ventral hypodermis; these cells are the descendants of three induced vulval precursor cells called Pn.p (n = 5, 6 and 7), they are linked by adherens junctions and they are embedded in hyp7. Heterotypic fusions between uninduced descendants of Pn.p (n = 3, 4 and 8) cells with hyp7 syncytium ($\mathbf{F_S}$) occur at this time. Other early cell fusions include longitudinal fusions ($\mathbf{F_L}$) between two **a** cells on each side before migrations and transverse fusions ($\mathbf{F_T}$) between two **c** cells on each side during the process of cell migration (Sharma-Kishore *et al.*, 1999). This stage is shown in Fig. 2A; for simplicity the four cells that form the central **f** ring (**vulF**) are shown as a cylinder with the anchor cell (**AC**) sitting dorsally. The anterior (left) and posterior **e** cells divide transversely (not shown) and migrate ventrally and laterally between the **vulF** and the hyp7 syncytium (not shown in the diagram; Fig. 2B). The next vulval ring is formed when the **d** cells migrate ventrally around the **e** ring (**vulE**) pushing dorsally the stack of **AC**, **vulF** and **vulE** (Fig. 2C). Following the same pattern of sequential and concerted radial short-range migrations around internal newly formed rings, coupled to stacking of rings an invagination is accomplished (Fig. 2D).

Final cell fusions and lumen formation

During the second stage the **AC** penetrates the centre of the **vulF** ring and then fuses with the dorsal utse uterine cell (not shown). This heterotypic fusion of the **AC+utse** ($\mathbf{F_{AC}}$) results in the poking of a hole necessary for the generation of a hollow tube that connects the dorsal uterus to the external ventral hypodermis through the vulva (Newman & Sternberg, 1996; Newman *et al.*, 1996; Sharma-Kishore *et al.*, 1999; Hanna-Rose & Han, 1999). Intratoroidal (within the rings) fusions of two types, longitudinal fusions ($\mathbf{F_{IL}}$) and transverse fusions ($\mathbf{F_{IT}}$), follow an invariant sequence forming only five syncytial rings because the cells within **vulB1** and **vulB2** do not fuse (Sharma-Kishore *et al.*, 1999).

Eversion and functional maturation

Vulval muscles attach to specific points on the epithelial rings, the vulval cuticle is secreted and eversion of the tube completes morphogenesis by forming a valve that is opened by contraction of the vulval muscles to allow egg-laying (Sharma-Kishore *et al.*, 1999).

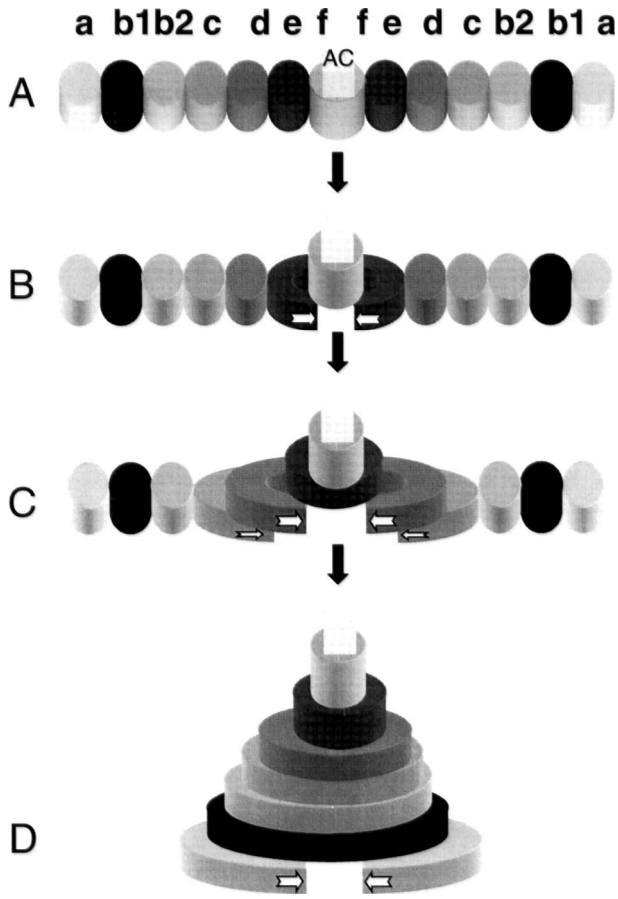

Fig. 2. *Ring formation as a morphogenetic force in vulva formation. Cell migrations around the central **f** cells, starting from the inner cells **e** to the outer cells **a** result in the formation of a stack of seven rings. A: Palindromic set of seven cells (**a-f + f-a**) in an anterior-posterior orientation arranged in two dimensions with the anchor cell (**AC**) sitting on the dorsal side of the **f** ring precursors; B: The **e** cells send filopodial extensions around and ventral to the **f** cells pushing them dorsally; C: The **d** cells send lateral and ventral extensions that surround the newly formed **vulE** ring and the **c** cells migrate ventrally surrounding the **d** cells; D: The last cells to migrate are the external **a** cells that will link the tube formed by a stack of seven rings to the hypodermis (Not shown here is the syncytial hypodermis (hyp7) that surrounds the vulva primordium; all the filopodial directed migrations occur between the inner rings and the hyp7 syncytium; the **AC** sends an extension ventrally that penetrates the **vulF** ring and will form the hole of the tube as the **AC** fuses to the **utse** uterine cell [not shown]; diagrams are with the anterior of the worm on the left-hand side of the page).*

Analyses of vulva formation in *Pristionchus pacificus* (Sommer & Sternberg, 1994a; Eizinger & Sommer, 1997; Jungblut & Sommer, 1998; Sigrist & Sommer, 1999), *Oscheius* sp. (Félix & Sternberg, 1997) and other nematodes (Sommer & Sternberg, 1994b; Sommer, 1997; Félix & Sternberg, 1998) where the induction of the vulva has been studied will help to understand how it is that ring formation, invagination and cell fusion generate diversity in nematodes.

A fusomorphogenetic hypothesis: inter- and intra-cellular membrane fusion drive morphogenesis in nematodes

Here a model for the role of membrane fusion during the generation of ring cells is postulated. It is proposed that redistribution of membranes is one of the forces responsible for elongation of the embryo and invagination of the vulva.

This model is based on the ultrastructural description of cell fusion intermediates in the embryonic hypodermis (Mohler *et al.*, 1998) and the male tail (Nguyen *et al.*, 1999), together with the detailed morphology of the cells during the cell fusion process (Podbilewicz & White, 1994; Newman *et al.*, 1996; Ch'ng & Kenyon, 1999; Nguyen *et al.*, 1999; Sharma-Kishore *et al.*, 1999). In this scenario, patterning of the embryo and the vulva are accomplished by the redistribution of membranes to different domains of the cells involved. Polarised recycling of membranes may couple cell fusion to vesicular transport and would account for the dynamic generation of ring shaped cells. These rings can easily elongate and dilate to control events like embryonic elongation, invagination of the vulva and exit of more than a thousand eggs of about 30 μm diameter through a tube composed of a stack of seven rings with a 10 μm diameter hole. This dilation of the tube of the vulva would be analogous to the intracellular transport of macromolecules across the nuclear pore complex (Gorlich & Mattaj, 1996).

The triggering of cell fusion by localised pore formation followed by expansion of the pore(s) and vesiculation of the lateral membranes in the dorsal hypodermis of the embryo (Mohler *et al.*, 1998) may result in a pathway of membrane recycling. Thus, the recycling of cell-fusion-derived-vesicles may be responsible for the polarised insertion of the membranes to the lateral domains of the hyp7 syncytial cell necessary for elongation of the embryo (Fig. 3). Each lateral transverse membrane would account for approximately 20 μm length by 2-3 μm depth of membranes. Since there are 16 junctions that fuse between the 17 dorsal cells in hyp7, if all these transverse membranes were inserted into the lateral membranes, this would be sufficient to elongate the embryo by 160 μm. Before elongation the length of the embryo is about 50 μm and at the end of morphogenesis there is a four-fold increase in length (Priess & Hirsh, 1986). Thus, redistribution of transversal dorsal membranes to the lateral plasma membrane of the growing hyp7 syncytial cell would save the cell from *de novo* synthesising two lateral membranes of about 200 μm each occupying a total area of about 800 μm^2.

The fusomorphogenetic hypothesis of vulva formation proposes how the five major rings of the *C. elegans* vulva become completely syncytial in the second phase of vulva development to redistribute lateral membranes from the boundaries of the component cells to the apical domain of syncytial rings. The proposed model involves the vesiculation of all the lateral membranes and the localised insertion to the luminal membrane by polarised exocytosis of all the vesicular carriers. This process would increase by two- to three-fold the surface area of the apical membranes facing the lumen. This increase in the luminal area may occur before the eversion of the vulva and would allow the dilation necessary for the release of the eggs in the adult. In particular the last intratoroidal fusions occur in **vulF** ring followed by **vulE**. These two rings have narrow lumens and before the last phase of vulva formation **vulE** makes attachments to the seam cells and the inner cuticle of the vulva starts to be secreted (Sharma-Kishore *et al.*, 1999). These exocytic events in the apical (luminal) domain and the formation of important attachments in the basal domain may be evidence for the redistribution of membranes through polarised secretion to the lumen and extensions towards the hypodermal seam cells. Eversion of the vulva involves plastic deformations of the rings to form a valve that is opened by the contraction of vulval and uterine muscles when eggs are laid; having extra-membrane reservoirs derived from fused lateral membranes between intercellular junctions may be a good strategy to accomplish these morphogenetic events. Mutations that affect glycosylation during the exocytic pathway result in abnormal invagination of the vulva (Herman & Horvitz, 1997, 1999; Herman *et al.*, 1999), supporting the involvement of membrane recycling and the exocytic pathway during morphogenesis of the vulva.

In summary, the fusomorphogenetic model (Fig. 3) is based on the following evidence: *i)* membrane fusion originates in discrete foci at or near the adherens junctions

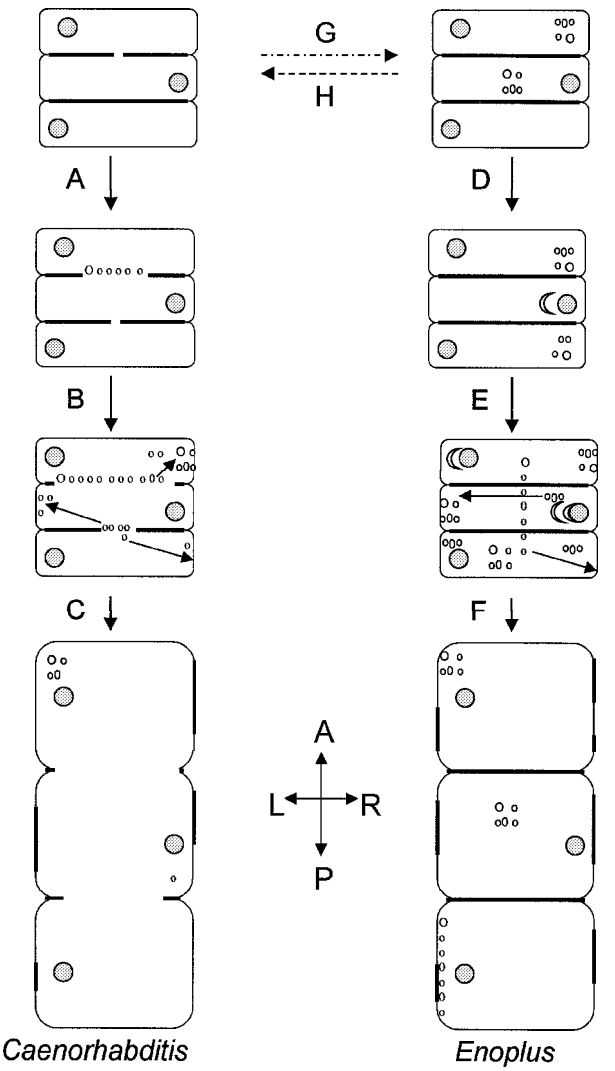

Fig. 3. *The fusomorphogenetic hypothesis. A: Three mononucleated cells fuse in an anterior to posterior sequence; a pore is formed between the membranes of the anterior pair of cells, the pore(s) dilate and then the membranes vesiculate; B: The posterior pair of cells fuse and follow the same ultrastructural steps: (1) pore formation; (2) dilation of the pore and (3) vesiculation of the fusing membranes; the vesicles are transported to the lateral membranes (arrows) of this small syncytium; C: Fusion of the vesicles to the lateral plasma membranes is coupled to a change in cell shape that results in the anterior-posterior elongation; D: Alternatively, cells can accomplish changes in shape without cell fusion by biosynthesis of new membranes through the secretory pathway or by the use of existing reserves or stocks of vesicular organelles that can fuse when needed; E: Crescent shaped organelles represent Golgi complex and the vesicles are targeted directly to the lateral membranes (arrows) where they fuse (F) in a polarised way. The specific insertion of newly synthesised membranes to the lateral domains of the plasma membrane (D-F) can bypass the necessity for cell fusion and membrane recycling (A-C). Since most cells have the potential of synthesising new membranes, (D-F) may be the default pathway. A combination of both strategies (A-C and D-F) and a balance between the two (G and H) involve membrane fusion as a requirement for morphogenesis. It is hypothesised that nematodes with syncytial hypodermis like* Caenorhabditis elegans *use cell fusion followed by polarised membrane recycling (A-C) and marine nematodes like* Enoplus brevis *use* de novo *synthesis of membranes coupled to polarised secretion. The fusomorphogenetic model predicts that specific mutations that disrupt the cell fusion machinery may be bypassed by the use of a higher biosynthetic pathway and polarised secretion (G). Two candidate mutations that reduce cell fusion in* C. elegans *have been recently characterised (Gattegno, Mohler, White & Podbilewicz, unpubl.). Modification in the cellular and molecular mechanisms that control the fusomorphogenetic balance (G and H) may be involved in the evolution of developmental mechanisms.*

(Podbilewicz & White, 1994; Mohler *et al.*, 1998; Nguyen *et al.*, 1999); *ii)* vesicles are formed as membranes fuse in the dorsal hypodermis (Mohler *et al.*, 1998) and in the male tail (Nguyen *et al.*, 1999); *iii)* cell fusions begin anteriorly and progress posteriorly (Podbilewicz & White, 1994; Mohler *et al.*, 1998; Nguyen *et al.*, 1999) and the relative increase in vacuolar volume is achieved in the same order (Nguyen *et al.*, 1999); *iv)* dramatic morphogenetic events in the hypodermis, vulva and male tail involve polarised increase in the plasma membrane surface coupled to polarised exocytosis as suggested by an increase in extracellular volume after the completion of cell fusions (Nguyen *et al.*, 1999).

The fusomorphogenetic model proposes testable hypotheses that are experimentally tractable by directly following the proposed recycling of membranes using membrane-localised Green Fluorescent Proteins, vital dyes like FM 4-64 and caged markers combined to fluorescence recovery after photobleaching. Using this technology it will be possible to label membranes and test whether membranes derived from cell-cell fusion are directly recycled to the lateral domains of syncytial cells.

Future perspectives

Evolution of cell fusion and ring formation

The fusomorphogenetic model also provides ways to explain how morphogenetic mechanisms may have e-

volved (Fig. 3). For example, many marine nematodes have cellular hypodermis (Chitwood & Chitwood, 1977) which means that cell fusion is not absolutely required in these species to accomplish elongation. This lack of cell fusion may be explained if the rate of membrane biosynthesis was very high in hypodermal cells of species with cellular hypodermis or alternatively if development was slower in such species which is the case for *Enoplus brevis* (Smith & Stephenson, 1970; Voronov & Panchin, 1998). Thus, redistribution of the lipids and proteins contained in the membranes from the dorsal transverse membranes to the longitudinal membranes could be bypassed by the *de novo* synthesis of new vesicles that can fuse in a polarised way to the lateral plasma membranes (Fig. 3). Thus, the fusomorphogenetic hypothesis may have evolved from cell-cell fusion followed by the recycling of vesicular membranes by polarised secretion in *Caenorhabditis* and related species to the synthesis of new membranes to be transported directly to the lateral plasma membranes of elongating marine nematodes like *Enoplus.*

Generation of diversity in nematodes and cell fusion. Male tail tip: How morphological diversity results from evolutionary changes

The tip of the male tail is a simple but elegant multicellular structure where cell fusion also plays a central role in morphogenesis (Baird *et al.*, 1991; Chow & Emmons, 1994; Fitch & Emmons, 1995; Nguyen *et al.*, 1999). In the *lep-1* mutants of *C. elegans* tail tip retraction fails forming a pointed ('leptoderan') adult male tail (Nguyen *et al.*, 1999). In these mutants the most posterior fusion between hyp10 and hyp9 cells does not occur. In a *Rhabditis* species, the anterior tail tip cells fuse, but hyp10 does not fuse and retraction does not occur mimicking the *lep-1* phenotype (Nguyen *et al.*, 1999). In the leptoderan species *Rhabditella axei* and *Oscheius myriophila* tail tip cells do not fuse and do not retract (Fitch, 1997). The results summarised here are a good example of how a detailed analysis of morphogenesis, genetics and evolution will give insights to understand the mechanisms responsible for the generation of diversity in nematodes (Emmons, 1997). Future work should concentrate on similar analyses regarding embryonic morphogenesis, the formation of the vulva and the role of cell fusion and ring formation in development across nematode species. At least two independent mutations that prevent cell fusion in the hypodermis have been recently isolated (Mohler, Gattegno, White and Podbilewicz, unpubl.) and their analysis will be helpful to test the morphogenetic hypothesis using molecular genetic approaches.

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