RESEARCH ARTICLE

Tubular Sprouting as a Mode of Vascular Formation in a Colonial Ascidian (Tunicata)

Fabio Gasparini,* Fabrizio Longo, Lucia Manni, Paolo Burighel, and Giovanna Zaniolo

Although phylogenetically related to vertebrates, invertebrate chordate tunicates possess an open circulatory system, with blood flowing in lacunae among organs. However, the colonial circulatory system (CCS) of the ascidian *Botryllus schlosseri* runs in the common tunic and forms an anastomized network of vessels, defined by simple epithelium, connected to the open circulatory system of the zooids. The CCS originates from epidermal evagination, grows, and increases its network accompanying colony propagation. New vessels are formed by means of mechanisms of tubular sprouting which, in their morphogenesis and molecular regulation, are very similar to those occurring in other metazoans, particularly during vertebrate angiogenesis. From the apex of new vessels, epithelial cells detach and migrate into the tunic, while exploring filopodia extend toward the tunic and possibly guide vessel growth. Immunohistology showed that growth factors fibroblast growth factor-2 and vascular endothelial growth factor and the receptor vascular endothelial growth factor receptor-1 participate in sprouting, associated with cell proliferation. As in vertebrates, these factors may regulate cell migration, proliferation, sprouting, and tube formation. Our data indicate that similar, conserved signals were co-opted in the sprouting processes of two nonhomologous circulatory systems, that of ascidian CCS, and vertebrate circulatory systems, by recruitment of the same signaling pathway. *Developmental Dynamics 236:719–731, 2007.*

Key words: angiogenesis; Botryllus schlosseri; cell migration; colonial circulatory system; growing vessel; growth factors; parallel evolution; proliferation; signaling pathway

Accepted 26 December 2006

INTRODUCTION

The process of branching, leading to the formation of both tubes (as in circulatory systems) and compact fibers (as in nerves; Carmeliet and Tessier-Lavigne, 2005), occurs frequently during the development of various organs in metazoans and is defined as sprouting. Several studies focusing on similarities in nerve and vessel sprouting (e.g., Carmeliet and Tessier-Lavigne, 2005; Suchting et al., 2006) and on the common mechanism of sprouting, suggest that invertebrates and vertebrates share a similar signaling pathway (Bulloch and Ridgway, 1989; Dent et al., 2003; Seipel et al., 2004; Gerhardt and Betsholtz, 2005; Holmes and Zachary, 2005).

In vertebrates, vascular sprouting has received particular attention because of its importance in physiological and pathological angiogenesis (Auguste et al., 2005). Angiogenesis is the process of blood vessel formation from existing vessels: ontogenetically, it occurs after vasculogenesis, i.e., the process giving rise to the heart and the first vascular net of embryo and annexes (Patan, 2000). Tubular sprouting is the most important angiogenic mechanism, and refers to the development and growth of new vessels starting from evagination of the endothelial wall. It follows various steps (Patan, 2000; Distler et al., 2003; Costa et al., 2004) and involves proliferation and migration of endothelial cells regulated by several growth factors, including VEGF (vascular endothelial growth factor, or VEGF-A) and bFGF (basic fibroblast growth factor,

Dipartimento di Biologia, Università degli Studi di Padova, Padova, Italy
Grant sponsor: Ministero della Università e Ricerca Scientifica e Tecnologica; Grant sponsor: Università degli Studi di Padova.
***Correspondence to: Fabio Gasparini, Dipartimento di Biologia, Università degli Studi di Padova, Via U. Bassi 58/B 35131 Padova, Italy. E-mail: fabio.gasparini@unipd.it**

DOI 10.1002/dvdy.21073
Published online 26 January 2007 in Wiley InterScience (www.interscience.wiley.com).

or FGF-2) which play key roles (Distler et al., 2003; Cao et al., 2004). FGF-2 induces angiogenesis and stimulates vessel growth; it has a strong mitogenic effect promoting proliferation, migration, and capillary tube formation from endothelial cells (Li et al., 2003; Fernandez, 2005; Zachary, 2005). VEGF is a critical inducer of proliferation, migration, sprouting, and tube formation in tissues during angiogenesis (Tammela et al., 2005). Its binding sites VEGFR-1/Flt-1 and VEGFR-2/Flk-1 work in a competitive way to regulate the VEGF signal in response to variations in physiological conditions (Ferrara et al., 2003; Kearney et al., 2004; Roberts et al., 2004; Ferrara, 2005; Marti, 2005; Nanka et al., 2006).

The signal cascades induced by growth factors produce the angiogenic stimulus, the local effect of which is degradation of the basement membrane, followed by endothelial cell migration and proliferation to form blind tubes (Risau, 1997; Kalluri, 2003; Auguste et al., 2005). The tips of newly forming vessels extend filopodia, which are used by the sprouts to sense and navigate, by means of a mechanism resembling axonal guidance (Gerhardt et al., 2003; Carmeliet and Tessier-Lavigne, 2005).

Notably, VEGF homolog and other angiogenic factors are involved in the development and maintenance of circulatory or other branching systems in several invertebrate taxa (de Eguileor et al., 2001; Cho et al., 2002; Tettamanti et al., 2003; Seipel et al., 2004; Gerhardt and Betsholtz, 2005). This finding suggests that the basic mechanisms of branching morphogenesis/tubular sprouting are well conserved and have been repeatedly recruited during the evolution of metazoans for the development of various organs.

For better understanding of the possible presence of conserved signaling cascades and the evolutionary origin of sprouting, we studied this process in ascidians. Due to their phylogenetic position at the base of chordates, the tunicates and their major representatives, the ascidians, are of interest in investigating aspects of vertebrate evolution, because similar mechanisms appear to have been recruited in extant chordates, starting from their common ancestor (Schubert et al., 2006). In addition, thanks to the relative simplicity of their structures, ascidians allow us to study developmental mechanisms that are much more complex in vertebrates. In this work, we used the ascidian *Botryllus schlosseri* to examine the sprouting mechanism that occurs during growth of the colonial circulatory system (CCS) and to compare it with that in other metazoans, particularly vertebrate angiogenic sprouting.

B. schlosseri is a colonial ascidian representing a model species for studies of developmental and evolutionary biology (reviewed in Manni and Burighel, 2006; Manni et al., 2006). Each colony derives from a swimming larva that metamorphoses into an oozooid, the founder of a new colony. Colonies are formed of numerous clonal individuals (blastozooids), originating by asexual reproductive cycles and organized in star-shaped systems. Each adult blastozooid bears a pair of buds (primary buds), which in turn, bear the successive generation of buds (secondary buds). The blastogenetic cycle lasts approximately 1 week at 18–20°C (Sabbadin, 1969) and ends with a takeover, during which adults stop filtering and degenerate, their buds open the siphons, becoming the new filtering adult generation, and the secondary buds become primary buds and produce a new bud generation (Lauzon et al., 2002; Cima et al., 2003; Tiozzo et al., 2006).

All the zooids are embedded in the common tunic, composed of a sort of thin extracellular matrix (ECM) containing numerous scattered cells. An intricate network of anastomized blood vessels runs throughout the tunic, ending in sac-like ampullae mainly located at the periphery of the colony. The ampullae take part in adhesion to the substrate and colony movement by elongating an active apex of thickened cells. They are also contractile and can help the hearts in maintaining blood flow (Brunetti and Burighel, 1969; Katow and Watanabe, 1978; Zaniolo, 1981). The CCS grows and participates in colonial asexual propagation, adapting and increasing its network to the demands of the growing colony (Brunetti and Burighel, 1969; Burighel and Brunetti, 1971). Its vessels are typically

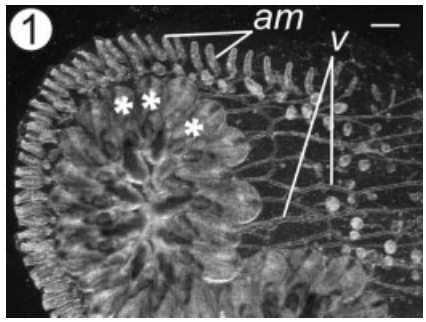


Fig. 1. Detail of a *Botryllus schlosseri* colony seen in vivo from the ventral side with zooids (asterisks) arranged in typical star-shaped system. The colonial circulatory system with anastomized vessels (v) and peripheral ampullae (am) extends into the thin transparent tunic. Scale bar = 0.3 cm.

defined by simple epithelium, and are in continuity with the internal open circulatory system running in the tissue lacunae of each zooid.

Our results on *B. schlosseri* colony propagation show that new vessels are formed by a mechanism of tubular sprouting from pre-existing vessels resembling sprouting mechanisms observed in other metazoans and during vertebrate angiogenesis.

RESULTS

In Vivo and Ultrastructural Observations

To examine the sprouting process, we first used light microscopy of living specimens. The thin tunic of *B. schlosseri* colonies adhering to glass slides is transparent, allowing easy observation of the network of colonial vessels with their ampullae and hemocytes (Fig. 1); it also allows us to select appropriate CCS regions for transmission electron microscopy observations. The results showed that CCS vessels are lined with a single epithelium, composed of cubic or flat cells, whose apical surface faces the tunic, whereas the basement membrane faces the lumen of vessels. Tight junctions (see below) are located apicolaterally (Fig. 2), so that the organization and polarity of the CCS is as follows: vessel lumen → basement membrane → epithelial cells → tunic.

This is the same polarity as that found in zooid epidermis, and CCS vessels do derive mainly as evaginations of blastozooid epidermis, when

radial vessels are formed as follows (Fig. 3): at takeover, the new primary buds extend a ventral epidermal evagination in the form of a blind tube, which grows toward the closest CCS vessel; at the same time, a similar tubular evagination moves from that vessel and elongates toward the first evagination; the two evaginations eventually meet each other and fuse, forming the radial vessel, connecting the bud to the CCS in the tunic (Figs. 3c,d).

We observed that a typical sprouting mechanism occurs during the formation of the two rudiments of the radial vessel. In addition, throughout the life of the colony, we identified several sprouts in the form of scattered evaginations of the CCS wall. They first appear as thickened regions of vessel wall, forming blind tubes, which either expand to give rise to ampullae or fuse with existing vessels, increasing network anastomosis (Figs. 4, 5). Ampullae develop from the tips of new vessels, whose lumen enlarges to form new sac-like structures (Fig. 6). This finding frequently happens behind the peripheral ring of ampullae during propagation and enlargement of the colony; later, the new ampullae elongate forward and are inserted in the peripheral ring.

Both in vivo and ultrastructural observations revealed that the apexes of new vessels are composed of thickened cells (Figs. 7, 8), which extend long filopodia into the surrounding tunic (Fig. 8). Cells have basal nuclei, well-developed rough endoplasmic reticulum, and many secretory membrane-bound granules (Fig. 9) discharging their contents into the tunic.

The blind vessel apex also appear to be the site of detachment of some epithelial cells that migrate toward the tunic (Figs. 4, 8, 10). Typically, epithelial cells have extended tight junctions recognizable at the apicolateral level and identifiable in thin sections as punctate cell to cell appositions between adjacent cells (Figs. 8 inset, 10). They present the typical feature upon transmission electron microscopy and correspond for position to the tight junction already observed both with conventional and freeze-fracture studies in several epithelia (epidermis included) of *B. schlosseri* and other ascidians (Georges, 1979; Lane et al., 1986; Martinucci et al., 1988). The migrating cells abandon the basement membrane and move from the epithelium, for a time maintaining their junctional connections with contiguous cells. The tight junctions then look like they shift from the apicolateral toward a basolateral position of the moving cells, as the latter progressively protrude into the tunic. The surface of moving cells contacting contiguous cells becomes progressively reduced, while the junctional belt of tight junctions reduces. Thus, the cells can finally detach without breaking the epithelium, while the contiguous cells close in to fill the space previously occupied by detaching cells (Figs. 8, 10, 11).

Thus, initial sprouts are observable as thick epithelial regions containing cells in the process of migration. There is no lumen in this first step (Fig. 4). Subsequently, when the thickened region evaginates, a lumen is present in the forming vessel, which has a single epithelium, although the tip of the epithelium appears to be multilayered, owing to the displacement of migrating cells and shifting of neighboring cells (see Fig. 10).

Proliferative Activity

The presence of proliferating cell nuclear antigen (PCNA) is considered to be evidence of cell proliferation. We used a fluorescent immunohistological approach on sections of *B. schlosseri* to reveal cell proliferation in the CCS. As positive control, an anti–α-tubulin antibody was used; as negative control, the primary antibody was omitted. Both controls gave the expected results: in negative controls, only red background fluorescence, given by Evans Blue treatment (see the Experimental Procedures section) was present in tissues (Fig. 12), whereas in positive controls, ciliary and cytoplasmic microtubules were strongly stained (Fig. 13). A search for blasts was also carried out, using the recognizing sequence (amino acids 111–125) of the antibody against human PCNA (Roos et al., 1993), to identify the homolog amino acid sequence in ascidians and other metazoans specifically recognized by the monoclonal PCNA antibody. Blast search revealed the presence of highly conserved sequences, particularly when ascidian and mammalian epitopes were compared (Fig. 14). To confirm these results, phylogenetic analysis was carried out, using complete amino acid sequences of PCNA protein. Ascidians clearly cluster with vertebrates in the neighbor-joining tree, bootstrap analysis being consistent (85%) with this topology (Fig. 15).

Experiments showed that PCNA was present in the apexes of new vessels; in contrast, the other regions of the circulatory system had rare labeled nuclei (Fig. 16). Double staining with 4′,6-diamidine-2-phenylidole-dihydrochloride (DAPI) indicated that not all the nuclei were in proliferation (Figs. 16b,d). It is noteworthy that the apical epithelium of elongated ampullae never showed PCNA, unlike the active apexes of new vessels. A few random blood cells also appeared stained.

Angiogenic Signals

The presence of FGF-2, VEGF, and receptor VEGFR-1 in the CCS was tested by an immunoperoxidase approach. The same positive and negative controls used for PCNA immunofluorescence were used, with similar results (Figs. 17, 18). In addition, because we used a primary antibody from rabbit in this approach, we introduced a supplementary negative control, which showed that rabbit antibodies do not react aspecifically with any tissues from *B. schlosseri* colonies. Because antibodies against FGF-2, VEGF, and VEGFR-1 gave equivalent responses in corresponding structures, labeled structures are now described for all antibodies.

All the antibodies marked most of the vessel sprouts, beginning from their first appearance as an epithelial region with cells evaginating toward the tunic (Fig. 19a). A positive response persisted at the apex of most of the forming vessels when they elongated and the lumen became recognizable (Figs. 19b,c).

A positive response was also identified in the active apical region of many peripheral ampullae (Fig. 19d–f), in some hemocytes, and in cells scattered in the tunic (Fig. 19c,d). During radial vessel formation, it was noteworthy that a positive response occurred in the anteroventral region of the bud

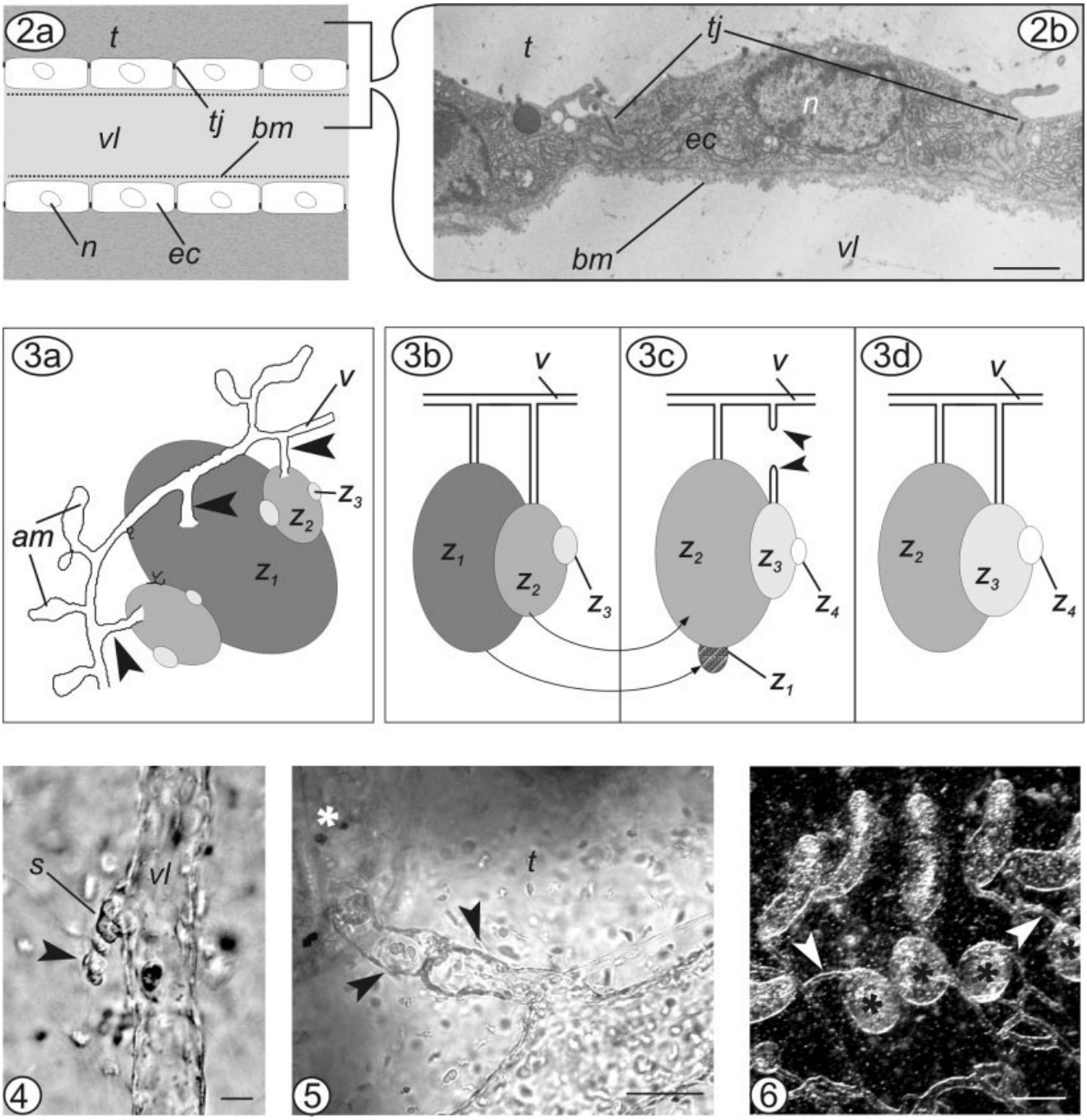


Fig. 2. Organization and basoapical polarity of colonial circulatory system (CCS) vessel epithelium. Basement membrane (bm) of epithelial cells (ec) face the vessel lumen (vl); apex touches tunic (t); tight junctions (tj) are in an apicolateral position. **a:** Scheme. **b:** Electromicrograph. n, nucleus. Scale bar = 1.8 μm in b.

Fig. 3. a: Scheme showing the circulatory relationship between zooids and the colonial circulatory system (CCS). Adult blastozooid (z_1) bears two primary buds (z_2), each with a pair of secondary buds (z_3). Note radial vessels (arrowheads) connecting zooids to CCS vessel (v). am, ampullae. b–d: Sketches showing blastogenetic cycle and radial vessel formation (arrowheads). Each zooid is identified by the same letters. **b:** Scheme of blastogenetic phase of Figure 3a. **c:** At takeover, adult zooid (z_1) regresses and its primary bud (z_2) becomes a new adult (arrows); while z_3 grows and becomes the new primary bud forming new budlets (z_4); at same time, rudiments of radial vessels (arrowheads) are formed by evagination of epidermis and vessel wall. **d:** Evaginated tubes of Figure 3c fuse to form fully developed radial vessel. v, CCS vessel.

Fig. 4. In vivo view of a sprout (s) starting as a swollen thick region of vessel epithelium. Note cells (arrowhead) detaching from sprout. vl, vessel lumen. Scale bar = 20 μm.

Fig. 5. Two new growing vessels (arrowheads) touch in the tunic (t) before fusing. The vessel on the left is a radial vessel originating from zooid (asterisk) epidermis; the vessel on the right derives from colonial circulatory system (CCS). Scale bar = 100 μm.

Fig. 6. Peripheral region of colonial circulatory system (CCS) as seen in vivo. Spherical ampullae (asterisks) lie below elongated ampullae and rise from tips of new vessels (arrowheads). Scale bar = 250 μm.

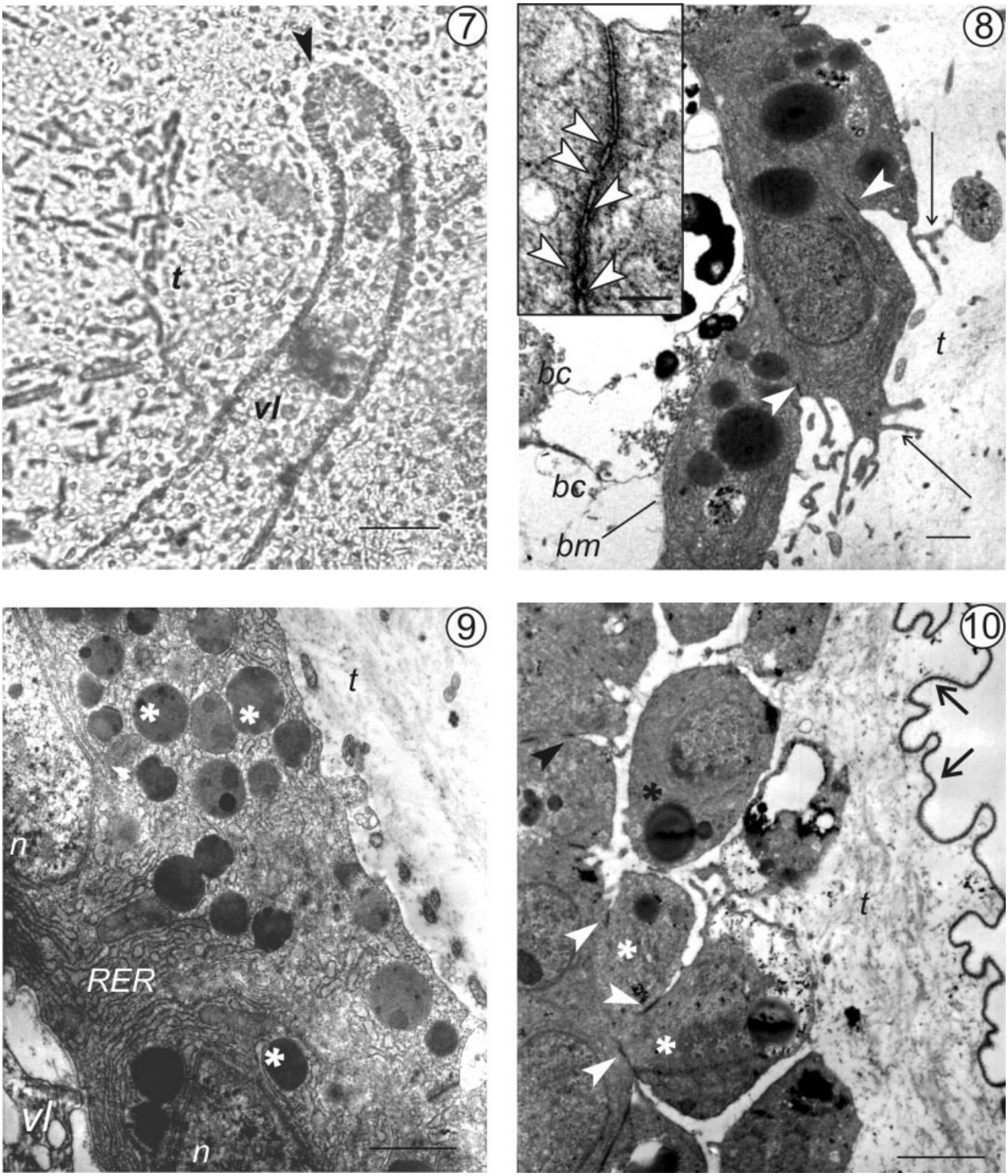


Fig. 7. A growing sprouting vessel with active apex epithelium (arrowhead), formed of thickened cells. The remaining vessel wall is formed of flat cells. vl, vessel lumen; t, tunic. Scale bar = 100 μm.

Fig. 8. Detail of active apex epithelium of a sprout. Several thin filopodia (arrows) extend into the surrounding tunic (t) from a protruding apex of cells. Tight junctions (white arrowheads) have an apicolateral position. Blood cells (bc) adhere to the basement membrane (bm). The inset shows the tight junction characterized by punctate cell to cell appositions (black bordered arrowheads). At these points, fusion of the outer half membrane leaflets of the adjacent cells occurs. Scale bars = 2 μm, 0.1 μm in inset.

Fig. 9. Electromicrograph of a growing vessel apex, whose cells have basal nuclei (n) and possess well-developed rough endoplasmic reticulum (RER) and many membrane-bound secretory granules (asterisks). vl, vessel lumen; t, tunic. Scale bar = 1 μm.

Fig. 10. Electromicrograph of a sprout region showing migrating cells. A cell appears free in the tunic (black asterisk). Others (white asterisks) are still attached to the epithelium by tight junctions (white arrowheads), displaced with respect to those (black arrowhead) of aligned epithelial cells. Arrows, external tunic surface; t, tunic. Scale bar = 2 μm.

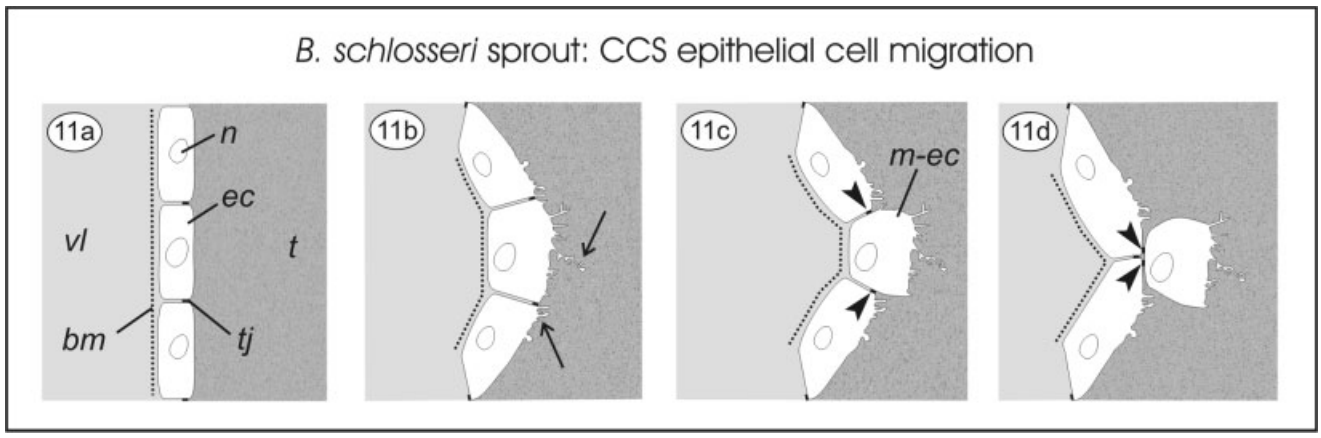


Fig. 11. Sketch of cell migration during sprouting in colonial circulatory system (CCS). **a:** Common aspects of epithelium: the basement membrane (bm) faces the vessel lumen (vl), the tunic (t) touches the apical surface of epithelium, and tight junctions (tj) are in apicolateral position. ec, epithelial cell; n, nuclei (see also Fig. 2). **b:** In the sprout apex, cells are thickened and form apical filopodia (arrows). **c,d:** During sprouting, a migrating epithelial cell (m-ec) protrudes toward the tunic, but the basement membrane maintains its continuity. Tight junctions (arrowheads) of migrating cells shift from an apicolateral position toward a basolateral position, while neighboring cells, which maintain the same junctions in the original apicolateral position, converge and touch each other below the migrating cells.

epidermis, precisely where the radial vessel began to evaginate (Fig. 19g–i). These positive responses to antibodies against factors and receptor were identified not only before epidermis evagination (Fig. 19i), but also in the apical regions of the two radial vessel rudiments, which, from bud and tunic vessel, elongate to meet and fuse to each other, to build up the radial vessel. It was noted that the apexes of growing vessels and ampullae sometimes did not show any response, whereas, scattered epithelial regions, without any recognizable morphological characteristic of sprout or active apex, were labeled.

DISCUSSION

Several investigations have shown that branching morphogenesis in epithelial tubules is an important feature of development for several organs in metazoans (e.g., Davies, 2002). Recent studies have revealed structural, mechanistic, and molecular analogies between angiogenic sprouting in vertebrates and the branching morphogenesis of the tracheas in the arthropod *Drosophila melanogaster*. *Drosophila* tracheas develop from clusters of ectodermal cells that invaginate and elongate to form primary tubes. Then other branches form and develop anastomoses, forming a network inside the body (Ghabrial et al., 2003; Myat, 2005). As in vertebrate angiogenesis, guidance of tracheal sprouts is mediated by tip cell filopodia, and the protein Branchless, a homolog of vertebrate FGF, plays a role resembling that of VEGF in vascular formation in vertebrates (Gerhardt and Betsholtz, 2005). Our results now show that branching morphogenesis also occurs during development of the circulatory system in a low chordate, by means of mechanisms that, despite some differences due to the characteristics of ascidian tissues, in particular the tunic, resemble the typical tubular sprouting of *Drosophila* tracheal development and vertebrate angiogenesis (Gerhardt and Betsholtz, 2005). These mechanisms involve extension of exploring filopodia, proliferation, and migration of cells, formation of lumen, and participation of angiogenic factors.

The present study was made possible by the specific characteristics of the circulatory system of the colonial ascidian *Botryllus schlosseri*. Indeed, like all other ascidians, this animal has an open circulatory component, running among organs in lacunae devoid of a continuous epithelium, but it also has a CCS, composed of vessels lined with a simple epithelium, running in the common tunic. Thus, the following characteristics make *B. schlosseri* an optimal model for studying aspects of tubular sprouting: (1) it can be cultured in the laboratory on glass slides; (2) its development can be followed in vivo under the microscope thanks to its transparency; (3) the CCS extends into the thin tunic, following a sort of bidimensional propagation, and all its variations during asexual reproduction can be observed without changing the physiological conditions; and (4) collecting pieces of the colony and experimental procedures can be carried out easily at selected developmental stages. In addition, thanks to the key phylogenetic position of *B. schlosseri* and other ascidians, data from investigations on these animals are crucial in understanding the mechanisms of morphogenesis and their evolution in chordates, hence, in vertebrates.

Histogenetic Mechanisms in *B. schlosseri* Sprouting

Cell proliferation.

Proliferative activity on the apex of new sprouting vessels in *B. schlosseri* resembles the process occurring in vertebrates, in which tubules, stimulated by several angiogenic factors, including FGF-2 and VEGF, grow, elongate, and proliferate to form lumen-containing vessels (Cross and Claesson-Welsh, 2001; Auerbach et al., 2003; Cross et al., 2003; Bohman et al., 2005). In vertebrates, studies of endothelial cell proliferation have provided evidence of the functional specialization of the tip cells from those of the stalk (i.e., endothelial cells surrounding the tips of

elongating vessels), with proliferation observed only in the stalk (Gerhardt and Betsholtz, 2005). In *B. schlosseri*, as the PCNA signal occurs all along the apex of sprouting vessels, it is not possible to identify a stalk-proliferative region versus a tip-nonproliferative region.

Filopodia and angiogenic signals.

In *B. schlosseri*, the CCS branches from short, blind evaginations of ex-

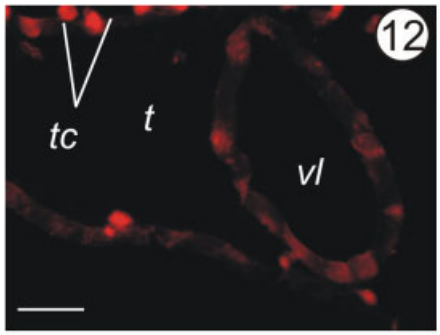


Fig. 12. A negative control for the immunofluorescence protocol with Evans Blue to turn background fluorescence of tissues red. The wall of one vessel and tunic cells (tc) but not tunic (t) display red fluorescence. vl, vessel lumen. Scale bar = 20 μm.

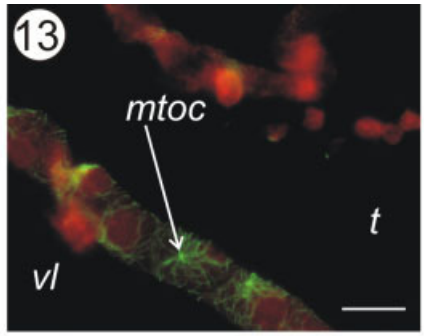


Fig. 13. A positive control for the immunofluorescence protocol with anti–α-tubulin antibody. In vessel cells, microtubules converge in microtubule organizing centers (mtoc). vl, vessel lumen. Scale bar = 13 μm.

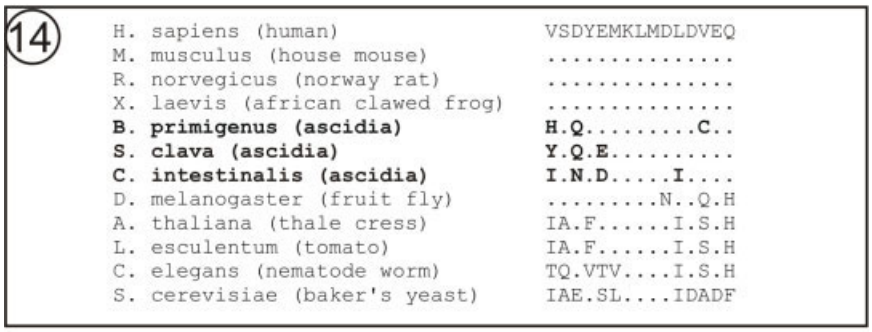


Fig. 14. Alignment of metazoan homolog amino acid sequences of the protein region recognized by antibody clone PC10 (Waseem and Lane, 1990) in *Homo sapiens* proliferating cell nuclear antigen (PCNA; Roos et al., 1993). Bold type indicates ascidian species and respective sequences. Sequences are conserved particularly between ascidian and mammal epitopes. GenBank codes: *Arabidopsis thaliana* NP_180517; *Botryllus primigenus* BAE47144; *Caenorhabditis elegans* AAC48257; *Drosophila melanogaster* P17917; *Homo sapiens* NP_872590; *Lycopersicon esculentum* CAD56690; *Mus musculus* NP_035175; *Rattus norvegicus* NP_071776; *Saccharomyces cerevisiae* AAB31034; *Styela clava* AAC37303; *Xenopus laevis* AAA49926. JGI code: *Ciona intestinalis* fgenesh3_pg.C_chr_12q000454.

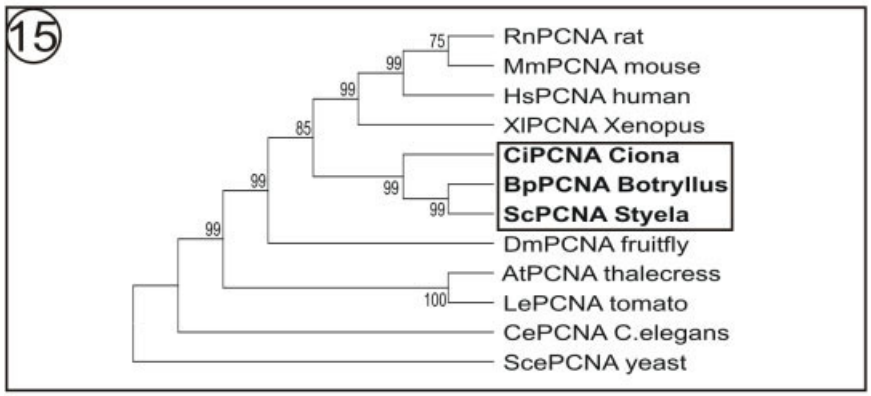
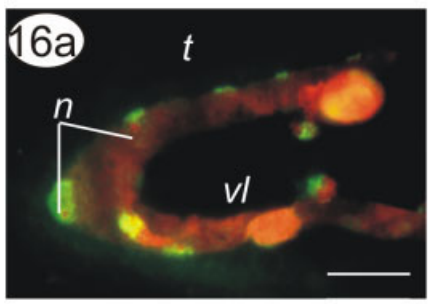
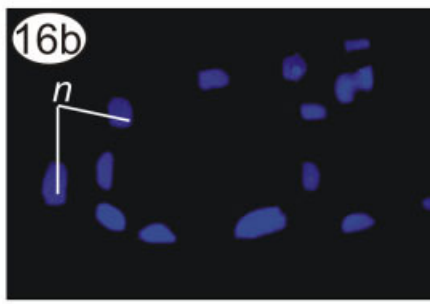
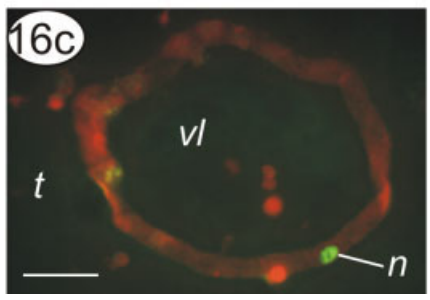


Fig. 15. Phylogenetic tree calculated using metazoan amino acid sequences of whole proliferating cell nuclear antigen (PCNA) proteins downloaded from GenBank and JGI (for codes see Fig. 14). Only topology is displayed. Bold type indicates ascidian species. Ascidians cluster with vertebrates (bootstrap analysis gives 85%).







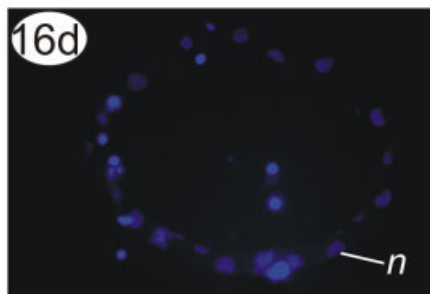


Fig. 16. Section of vessels treated with the immunofluorescence protocol for proliferating cell nuclear antigen (PCNA) staining. **a,b:** A longitudinal section of a growing vessel apex. In a, left, the tip of the sprout is thick and has stained green nuclei (n) positive to anti-PCNA. In b, the same section shows all nuclei stained blue by DAPI. Not all nuclei react to anti-PCNA. **c,d:** A transverse section of a vessel far from the growing apex. In c, only one nucleus (n) is positive to anti-PCNA. In d, the same section shows the nuclei stained blue by DAPI. t, tunic; vl, vessel lumen. Scale bars = 15 μm in a, 35 μm in c.

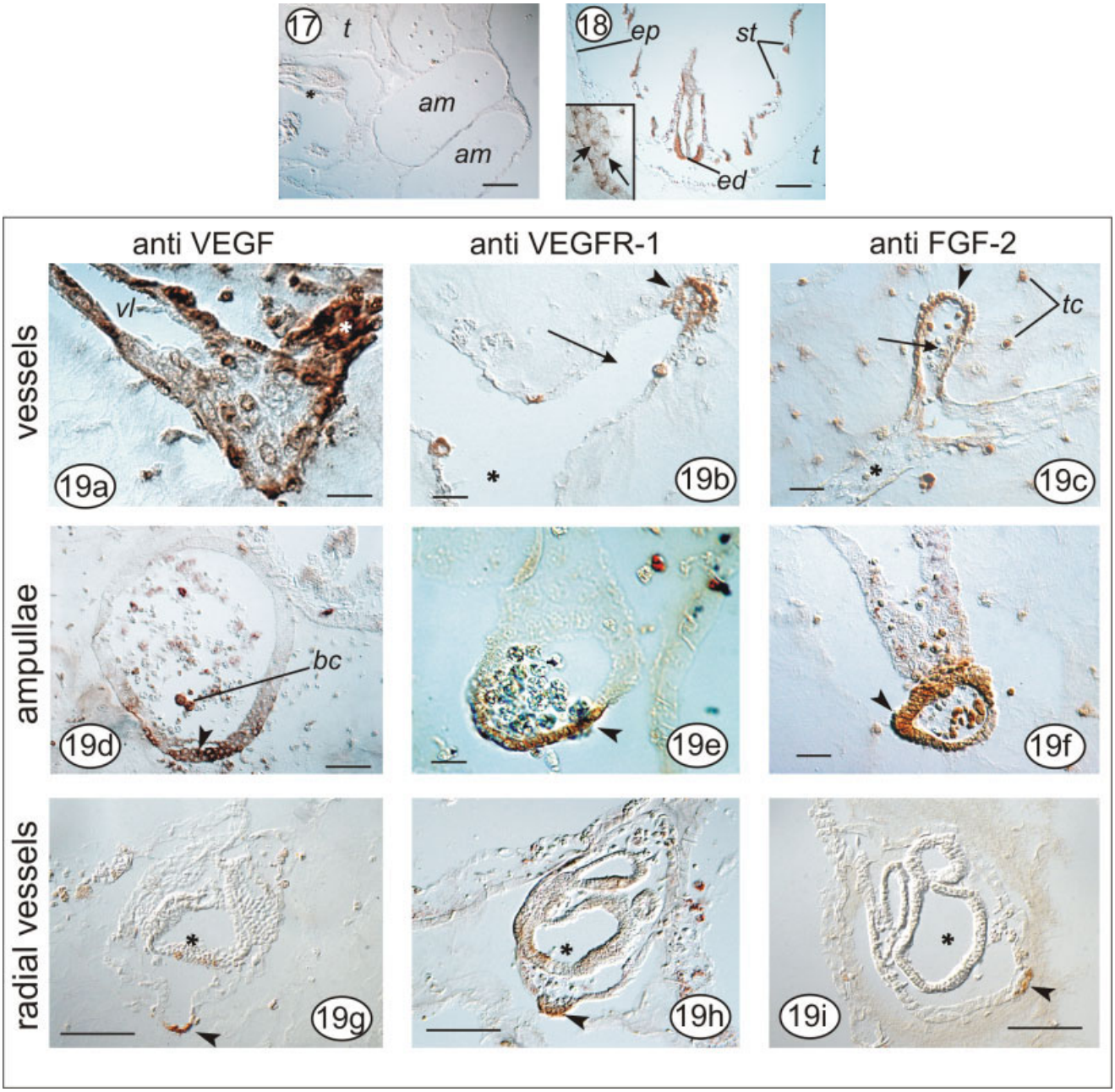


Fig. 17. Negative control for the immunoperoxidase protocol. The section is at the level of ampullae (am) and a zooid (asterisk). All structures are diaphanous. t, tunic. Scale bar = 120 μm.

Fig. 18. Positive control for the immunoperoxidase protocol with anti–α-tubulin antibody. Transverse section of an adult zooid. Cilia and microtubules are highlighted in brown. Insert: section of vessel epithelium with stained cell (arrows). ed, endostyle; ep, zooid epidermis; st, stigmata; t, tunic. Scale bar = 100 μm.

Fig. 19. Panel with sections of colonies treated with antibodies anti-vascular endothelial growth factor (VEGF), anti–VEGF receptor-1 (VEGFR-1) and anti-fibroblast growth factor-2 (FGF-2); reactions to antibodies are highlighted in brown. Photos taken from experiments in which the same antibody was used are ordered in columns (starting from left: anti-VEGF, anti–VEGFR-1, anti–FGF-2). Response of specific structures (sprouting vessels, ampullae, sprouting radial vessels) to antibodies are shown in three rows, respectively. **a:** A longitudinal section of a small vessel from which a sprout (asterisk) is forming and has strongly marked cells (note that this initial sprout lacks a lumen). vl, vessel lumen from which the sprout departs. **b,c:** A longitudinal sections of elongating new vessels (arrows), which depart from pre-existing ones (asterisks) and show reaction to antibodies (arrowheads). In c, some tunic cells are labeled (tc). **d–f:** Ampullae with apexes showing response to antibodies (arrowheads). In d, some labeled blood cells are clearly visible (bc). **g–i:** Sections of buds (asterisks) with epidermis evaginating to form radial vessels, with tip cells positive to antibodies (arrowheads). Note: the epidermal bud region from which vessels originate is marked at the onset of formation (h,i); later, when the vessel elongates, label persists in the apical region (g). Scale bars = 20 μm in a–f, 50 μm in g–i.

isting vessels. The tip cells of these evaginations constitute the active apex and extend filopodia toward the tunic.

Sprouting studies have shown that *Drosophila* tracheae and vertebrate blood vessels are guided by means of filopodial projections (Gerhardt et al., 2003; Davies, 2005). These cytoplasmic extensions probably guide the branches in the same way as filopodia guide neural growth cones (Lawson and Weinstein, 2002). In *B. schlosseri* CCS tip cells, the filopodia that advance into the tunic probably have an equivalent function and, by "tasting the soil," chemically attracted by factors, guide and orientate vessel growth. This hypothesis is strongly supported by the immunohistological finding that VEGF, VEGFR-1, and FGF-2, which play a role in the signaling pathways occurring in branch sprouting, particularly in vertebrate angiogenic sprouting (Distler et al., 2002; Ghabrial et al., 2003; Warburton et al., 2005), are also present in the sprouting structures of *B. schlosseri*. Thus, our data agree with the idea that these conserved signals were co-opted during the evolution of branched structures. Indeed, it appears that ascidian vessels too, like vertebrate vessels (which arose later in evolution than nerves), co-opted the same evolutionarily conserved guidance cues that help axons to navigate to their targets (Suchting et al., 2006).

We observed that VEGF, VEGFR-1, and FGF-2 are distributed in most (but not all) of the sprouting structures of *B. schlosseri* CCS, and sometimes also in normal epithelial regions that do not appear to be involved in vessel formation. The absence of positive responses in some sprouting structures may be associated with the noncontinuous intense expression of each signal during the sprouting process. Conversely, the rare absence of the signal may also be related to regression of the vessel or a block in its growth. Alternatively, the occurrence of the signal in scattered regions of the normal epithelium may indicate early molecular activation of cells before morphological changes toward sprouting, and/or low, progressive production of signals for the physiological maintenance of CCS activity and development.

FGF-2, VEGF, and VEGFR-1 appear to have an equivalent distribution in corresponding CCS structures, and this finding agrees with the known autocrine and paracrine roles of the two factors and their synergic induction of angiogenesis (Costa et al., 2004; Hamden et al., 2005; Bikfalvi et al., 1997). Of particular interest is the finding that VEGF and VEGFR-1 are expressed at the level of the same territory.

Cell migration and basement membrane.

Some epithelial cells detach from the tips of growing vessels and migrate toward the tunic. The migration of cells out of the epidermis requires a reorganization of the cytoskeleton in such a way that cells lose their polarity and abandon the attachment to the basement membrane and to the contiguous cells. However, during the cytoplasmic extension toward the tunic, in the migrating cells, the tight junctions are not rapidly disassembled, but, although progressively reduced in area, they maintain their contact with junctional regions of contiguous cells. So the epithelial integrity is maintained during the whole process of migration and detachment of the cells, as evidenced also during other ascidian morphogenetic mechanisms as fusion and perforation of various epithelia (Manni et al., 2002). The detachment of cells from *B. schlosseri* CCS sprouts finds a correspondence to angiogenic sprouts of vertebrates at the tip of which detachment of endothelial cells toward the surrounding stroma occurs (Distler et al., 2003). However, a striking difference appears when comparing vertebrates and ascidians. In vertebrates, the basement membrane lies between stroma and endothelial cells, and its degradation accompanies the process of cell migration, allowing the elongation of endothelial filopodia and facilitating their contact with angiogenic factors sequestered in the ECM to direct cell migration (Parker et al., 2002; Kalluri, 2003; Sund et al., 2004). In *B. schlosseri*, the detachment and migration of tip cells is not associated with basement membrane degradation, because the vessel epithelium has reversed basoapical polarity with respect to vertebrate endothelium. Its vessels originate from tubular evagination of epidermis or pre-existing vessels: the basement membrane faces the vessel lumen and the apical plasmalemma faces the tunic (Fig. 20a,a^{I}). Thus, cells migrating from epithelium to tunic do not cross the basement membrane barrier (Fig. 11), as opposed to vertebrate endothelial cells, in which the basement membrane, facing the ECM, acts as a barrier to cell migration.

Lumen formation.

Another difference between vertebrate and ascidian sprouting related to cell polarity regards the basement membrane of new vessel endothelium/epithelium and its relationship with the process of lumen formation (Fig. 20). Vertebrate sprouts possess a tip region in which the endothelial cells are organized as a compact string without a lumen (Patan, 2000). Subsequently, the sprouts mature into new vessels with lumen, and a continuous basement membrane is formed (Davis and Senger, 2005; Fig. 20a–d). The integrity of the basement membrane appears necessary to guarantee normal development and to protect the endothelium against mechanical stress caused by blood flow pressure. In mice, deletion experiments of typical basement membrane molecules (e.g., Collagen type IV) causes embryonic mortality when defects involve cells exposed to pressure, as occurs in the heart and blood vessels (Poschl et al., 2004; Davis and Senger, 2005). At the sprout tips, even though protection of the basement membrane is lacking, compact strings of endothelial cells can compensate for it. In *B. schlosseri*, we observed that cavitation extends to the tips of sprouting vessels, and the basement membrane always maintains its integrity (Figs. 11, 20a^{I},b^{I},c^{I},d^{I}). Thus, with respect to vertebrates, the sprouting mechanism of *B. schlosseri* is apparently simpler, because there is a persistence of basement membrane that may protect tip cells from mechanical stress.

The role of the tunic.

The tunic is a urochordate structure unique among metazoans. It constitutes a connective-like tissue (Peres, 1948), produced by the epidermis on the body surface, with collagen (Patri-

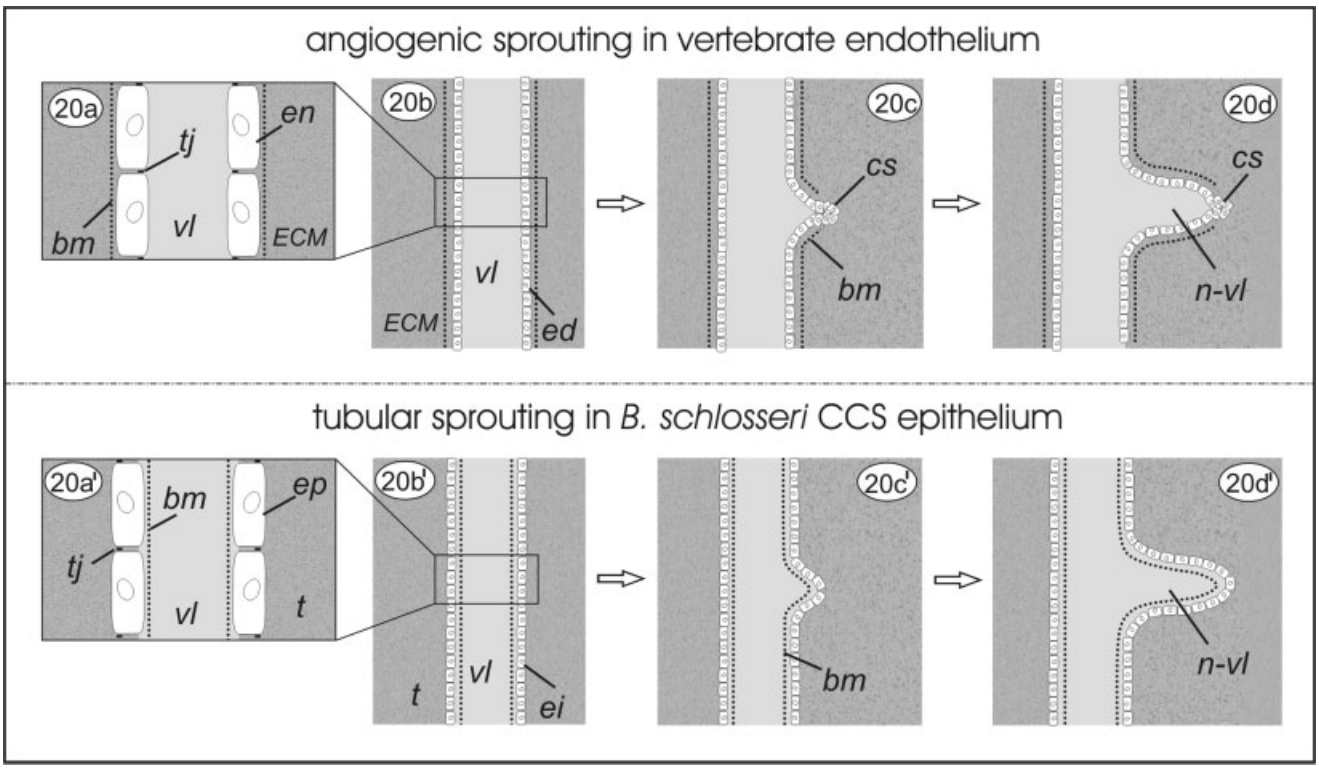


Fig. 20. Sketch comparing vertebrate angiogenic sprouting (a–d) with the *Botryllus schlosseri* colonial circulatory system (CCS) tubular sprouting (20a^{l},b^{l},c^{l},d^{l}). **a–a^{l},b–b^{l}:** Drawings of inactive vessels; 20a,a^{l}: enlargements of 20b,b^{l}. Note the different positions of tight junctions (tj) and basement membranes (bm), focusing on the inverse polarity of vertebrate endothelium (en) with respect to ascidian epithelium (ep). ECM, extracellular matrix; vl, vessel lumen; t, tunic. **c–d:** In vertebrates, the sprout tip forms a compact string (cs) without a lumen and the basement membrane is lacking; during vessel elongation in sprouting, a lumen forms behind the tip (n-vl) and a basement membrane is re-established. **c^{l}–d^{l}:** In *B. schlosseri*, a lumen always reaches the tip of the sprout, and the vessel elongates, maintaining a basement membrane as far as the tip of the sprout.

colo and Ferrarella, 1973; Vizzini et al., 2002) and cellulose components (De Santo, 1968; Matthysse et al., 2004; Nakashima et al., 2004) in which isolated cells are embedded (Burighel and Cloney, 1997). Therefore, epidermal cells are located between the external matrix (tunic) and the basement membrane. As previously discussed, in vertebrates and most metazoans, the typical epidermis organization has reverse polarity (starting from outside the organism: epithelial cells → basement membrane → lower ECM). In other metazoans, as in insects, peripheral hemolymphatic lacunae are bounded directly by the basement membrane of the epidermis, which produces an ECM (cuticle) on the surface of the body wall (Harrison and Locke, 1999), resembling the same organization and polarity as the epidermis in urochordates. However, the cuticle never incorporates both connective elements and isolated cells, and it is made up of an amorphous, rigid substance (chitin).

Thus, the peculiarity of the tunic in *B. schlosseri* and Urochordata seems to lie in the type of external matrix, not only because it contains cellulose, but also because it is collagenous and populated by several cells. The apical plasmalemma in the epithelial cells of *B. schlosseri* vessels are exposed directly to the collagenous stroma, and we assume that factors regulating vessel sprouting are collagens and other molecules embedded in the tunic. This mechanism may parallel the case of angiogenic sprouting in vertebrates, which is activated by ECM after basement membrane degradation, with subsequent exposure of the endothelial plasmalemma to angiogenic factors (Davis and Senger, 2005). Thus, our results support the idea that, in metazoans, the basement membrane serves not only to maintain the integrity of epithelia, but also to separate the epithelial cell membrane from interstitial factors dispersed in the ECM. Except in ascidians, the degradation of the basement membrane is necessary to expose cells to interstitial factors, activating the signaling pathways that drive sprouting morphogenesis.

CONCLUSION

The wall of *B. schlosseri* CCS is unlike vertebrate endothelium, in embryonic origin (the former is ectodermic, the latter mesodermic) and epithelial polarity. However, in *B. schlosseri*, as in vertebrate angiogenesis, new vessels form by sprouting, and in both cases the process involves filopodia elongation, cell proliferation and migration, and the participation of homolog factors as FGF-2 and VEGF and the receptor VEGFR-1, which play a critical role in sprout growth and tube formation.

This report is the first to indicate the occurrence of the same histogenetic mechanisms and homologous molecules in the development of vessels in vertebrates and ascidians. In addition, all our data on *B. schlosseri* CCS support the view that parallel evolution may have occurred in the

sprouting process of two nonhomologous (i.e., ascidian CCS and vertebrate circulatory) systems, with the recruitment of the same signaling pathway, during the origin and differentiation of urochordates and vertebrates.

EXPERIMENTAL PROCEDURES

In Vivo and Ultrastructural Procedures

B. schlosseri forms flat, variously pigmented colonies, extending on submerged vegetal and rocky substrates. Colonies, originally collected from the Lagoon of Venice, were cultured in the laboratory at 18°C, according to Sabbadin's technique (Sabbadin, 1955, 1960). Colonies adhere to and grow on glass slides, and this makes them easy to observe in vivo under a microscope (Fig. 1). In vivo observation was carried out by means of both Leica MZ75 stereomicroscopy (with image acquisition by a Leica DC100 digital photo camera) and Leica DMR light microscopy (with image acquisition by a Leica DFC 480 digital photo camera).

Transmission electron microscopic analysis was carried out on colonies prepared with the following procedure: small colonies were anesthetized with MS222, detached from the glass with a blade, and 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, specimens were dehydrated and embedded in Epon 812. Thanks to the transparency of the resin, specimens could be oriented before cutting. Series of thick sections (1 μm) were stained with toluidine blue and observed to check appropriate levels for preparing ultrathin sections (60 nm), which were given contrast by staining with uranyl acetate and lead citrate. Photomicrographs were taken with a Hitachi H-600 electron microscope operating at 80 V.

Immunohistology

Anesthetized colonies were fixed overnight at 4°C in freshly prepared Bouin fixative, dehydrated in graded series of ethanol to absolute ethanol and xylene, and embedded in Paraplast (Sherwood Medical). Samples were sectioned (7 μm), cleaned from Paraplast by xylene (15 min), incubated for 30 min with a 6% H_2O_2 solution in methanol to eliminate endogenous peroxidase (only immunoperoxidase protocol), rehydrated in a graded series of ethanol to phosphate buffered saline (PBS), and immediately used. Antibodies were diluted in a solution of 0.5% bovine serum albumin (BSA) in PBS.

To check proliferative activity, an immunofluorescence protocol (IFP) was applied with a mouse anti-PCNA (Clone PC10) monoclonal antibody (Sigma, 1:500 dilution), which recognizes an epitope conserved from man to fission yeast (Waseem and Lane, 1990). As secondary antibody, a goat anti-mouse immunoglobulin G (IgG), fluorescein isothiocyanate–linked whole antibody (Calbiochem), 1:150 dilution, was used. Sections were pretreated with Evans Blue to turn the natural background fluorescence of tissues red.

Antibodies against vertebrate VEGF, VEGFR-1, and FGF-2 were used to test their presence with an immunoperoxidase protocol (IPP). Sections were incubated with primary antibodies at the following dilutions: rabbit anti-human $VEGF_{165}$, polyclonal antibody (Oncogene, 1:100 dilution); rabbit anti-human VEGFR-1 (flt-1), polyclonal antibody (Santa Cruz, 1:300 dilution); and rabbit anti-human FGF2, polyclonal antibody (Oncogene, 1:35 dilution). As secondary antibody, a donkey anti-rabbit IgG, horseradish peroxidase (HRP) -linked whole antibody (Amersham Biosciences), 1:100 dilution, was used.

Rehydrated sections were processed as follows: Triton X-100, 0.5% in PBS (15 min); Trypsin, 0.1% in 0.01% CaCl in PBS (12 min; only Anti-PCNA and Anti-VEGF); Evans Blue 1% in PBS (Sigma; 15 min; only IFP); BSA 1% in PBS (90 min); incubation with primary antibody (overnight at 4°C); incubation with secondary antibody (90 min at 37°C); treatment with 3,3′-diaminobenzidine, 0.7 mg/ml in Urea H_2O_2 1.6 mg/ml Tris buffer 0.06 M (Sigma *Fast*; 3 min 30 sec; only IPP); 1 μg/ml DAPI (Sigma; 4 min; only IFP). Samples were then mounted in Vectashield (Vector) for IFP, but dehydrated in graded series of ethanol and a final step of xylene (15 min) and mounted in Eukitt (Electronic Microscopy Sciences) for IPP. Sections were photographed with a Leica DMR light microscope accessorized with both fluorescence equipment and Normansky interference contrast optics and a Leica DFC 480 digital photo camera.

As positive control, a mouse anti–sea urchin α-tubulin monoclonal antibody (Sigma, 1:10,000 dilution) was used for each experiment. For negative controls, two different approaches were used: (1) a solution of 0.5% BSA in PBS, instead of primary antibody solution, was used in a section of each experiment; (2) a rabbit anti-goat IgG, HRP-linked whole antibody (Dakocytomation, 1:150 dilution) was used as primary antibody, omitting the secondary antibody step, to verify the absence of aspecific reactions by primary antibodies from rabbit. We did not use the same procedure with an anti-IgG from mouse, because both the nuclear (mouse anti-PCNA) and filamentous (mouse anti–α-tubulin) responses of anti-mouse primary antibody used for experiments gave good indications about their specificity. In addition, the monoclonal origins of the antibodies and the high molecular conservation in metazoans of the recognized molecules (Waseem and Lane, 1990; Tuszynski et al., 2006) support the specificity of their antigen recognition.

Molecular Phylogeny

All sequences were downloaded from Genbank or JGI. Alignment was constructed using CLUSTALX (Thompson et al., 1997) and refined by eye. A phylogenetic tree was built using the neighbor-joining method (Saitou and Nei, 1987) with the MEGA3 program (Kumar et al., 2004). Bootstrap analysis was carried out for phylogenetic analysis: 5,000 iterations (Felsenstein, 1992).

ACKNOWLEDGMENTS

The authors thank M. Del Favero, R. Mazzaro, and C. Friso for technical help. This study was founded by grants from the Ministero della Università e Ricerca Scientifica e Tecnologica and by the Università degli Studi di Padova to G.Z., L.M., and P.B.

REFERENCES

Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N. 2003. Angiogenesis assays: a critical overview. Clin Chem 49:32–40.

Auguste P, Lemiere S, Larrieu-Lahargue F, Bikfalvi A. 2005. Molecular mechanisms of tumor vascularization. Crit Rev Oncol Hematol 54:53–61.

Bikfalvi A, Savona C, Perollet C, Javerzat S. 1998. New insights in the biology of fibroblast growth factor-2. Angiogenesis 1:155–173.

Bohman S, Matsumoto T, Suh K, Dimberg A, Jakobsson L, Yuspa S, Claesson-Welsh L. 2005. Proteomic analysis of vascular endothelial growth factor-induced endothelial cell differentiation reveals a role for chloride intracellular channel 4 (CLIC4) in tubular morphogenesis. J Biol Chem 280:42397–42404.

Brunetti R, Burighel P. 1969. Sviluppo dell'apparato vascolare coloniale in *Botryllus schlosseri* (Pallas). Pubbl Staz Zool Napoli 37:137–148.

Bulloch AG, Ridgway RL. 1989. Neuronal plasticity in the adult invertebrate nervous system. J Neurobiol 20:295–311.

Burighel P, Brunetti R. 1971. The circulatory system in the blastozooid of the colonial ascidian *Botryllus schlosseri* (Pallas). Boll Zool 38:273–289.

Burighel P, Cloney RA. 1997. Urochordata: Ascidiacea. In: Harrison FW, Ruppert EE, editors. Microscopic anatomy of invertebrates. Vol. 15. Hemichordata, Chaetognatha, and the invertebrate chordates. New York: Wiley-Liss, Inc. p 221–347.

Cao R, Eriksson A, Kubo H, Alitalo K, Cao Y, Thyberg J. 2004. Comparative evaluation of FGF-2-, VEGF-A-, and VEGF-C-induced angiogenesis, lymphangiogenesis, vascular fenestrations, and permeability. Circ Res 94:664–670.

Carmeliet P, Tessier-Lavigne M. 2005. Common mechanisms of nerve and blood vessel wiring. Nature 436:193–200.

Cho NK, Keyes L, Johnson E, Heller J, Ryner L, Karim F, Krasnow MA. 2002. Developmental control of blood cell migration by the *Drosophila* VEGF pathway. Cell 108:865–876.

Cima F, Basso G, Ballarin L. 2003. Apoptosis and phosphatidylserine-mediated recognition during the take-over phase of the colonial life-cycle in the ascidian *Botryllus schlosseri*. Cell Tissue Res 312:369–376.

Costa C, Soares R, Schmitt F. 2004. Angiogenesis: now and then. Apmis 112:402–412.

Cross MJ, Claesson-Welsh L. 2001. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. Trends Pharmacol Sci 22:201–207.

Cross MJ, Dixelius J, Matsumoto T, Claesson-Welsh L. 2003. VEGF-receptor signal transduction. Trends Biochem Sci 28: 488–494.

Davies JA. 2002. Do different branching epithelia use a conserved developmental mechanism? Bioessays 24:937–948.

Davies JA. 2005. Watching tubules glow and branch. Curr Opin Genet Dev 15: 364–370.

Davis GE, Senger DR. 2005. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ Res 97:1093–1107.

de Eguileor M, Grimaldi A, Tettamanti G, Ferrarese R, Congiu T, Protasoni M, Perletti G, Valvassori R, Lanzavecchia G. 2001. *Hirudo medicinalis*: a new model for testing activators and inhibitors of angiogenesis. Angiogenesis 4:299–312.

De Santo RS. 1968. The histochemistry, the fine structure, and the ecology of the synthesis of the test in *Botryllus schlosseri* (Pallas) Savigny. Ph.D. Dissertation. New York: Columbia University.

Dent EW, Tang F, Kalil K. 2003. Axon guidance by growth cones and branches: common cytoskeletal and signaling mechanisms. Neuroscientist 9:343–353.

Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. 2003. Angiogenic and angiostatic factors in the molecular control of angiogenesis. Q J Nucl Med 47:149–161.

Distler O, Neidhart M, Gay RE, Gay S. 2002. The molecular control of angiogenesis. Int Rev Immunol 21:33–49.

Felsenstein J. 1992. Estimating effective population size from samples of sequences: a bootstrap Monte Carlo integration method. Genet Res 60:209–220.

Fernandez B. 2005. Arterialization, coronariogenesis and arteriogenesis. In: Clauss M, Breier G, editors. Mechanisms of angiogenesis. Basel, Switzerland: Birkhauser Verlag. p 53–63.

Ferrara N. 2005. The role of VEGF in the regulation of physiological and pathological angiogenesis. In: Clauss M, Breier G, editors. Mechanisms of angiogenesis. Basel, Switzerland: Birkhauser Verlag. p 209–231.

Ferrara N, Gerber HP, LeCouter J. 2003. The biology of VEGF and its receptors. Nat Med 9:669–676.

Georges D. 1979. Gap and tight junctions in tunicates. Study in conventional and freeze-fracture techniques. Tissue Cell 11: 781–792.

Gerhardt H, Betsholtz C. 2005. How do endothelial cells orientate? In: Clauss M, Breier G, editors. Mechanisms of angiogenesis. Basel, Switzerland: Birkhauser Verlag. p 3–15.

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C. 2003. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 161:1163–1177.

Ghabrial A, Luschnig S, Metzstein MM, Krasnow MA. 2003. Branching morphogenesis of the *Drosophila* tracheal system. Annu Rev Cell Dev Biol 19:623–647.

Hamden KE, Whitman AG, Ford PW, Shelton JG, McCubrey JA, Akula SM. 2005. Raf and VEGF: emerging therapeutic targets in Kaposi's sarcoma-associated herpesvirus infection and angiogenesis in hematopoietic and nonhematopoietic tumors. Leukemia 19:18–26.

Harrison FW, Locke E. 1999. Microscopic anatomy of invertebrates. Vol. 11A. Insecta. New York: Wiley-Liss, Inc. 496 p.

Holmes DI, Zachary I. 2005. The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease. Genome Biol 6:209.

Kalluri R. 2003. Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer 3:422–433.

Katow H, Watanabe H. 1978. Fine structure and possible role of ampullae on tunic supply and attachment in a compound ascidian, *Botryllus primigenus* OKA. J Ultrastruct Res 64:23–34.

Kearney JB, Kappas NC, Ellerstrom C, DiPaola FW, Bautch VL. 2004. The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. Blood 103:4527–4535.

Kumar S, Tamura K, Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5: 150–163.

Lauzon RJ, Ishizuka KJ, Weissman IL. 2002. Cyclical generation and degeneration of organs in a colonial urochordate involves crosstalk between old and new: a model for development and regeneration. Dev Biol 249:333–348.

Lane NJ, Dallai R, Burighel P, Martinucci GB. 1986. Tight and gap junctions in the intestinal tract of tunicates (Urochordata): a freeze-fracture study. J Cell Sci 84:1–17.

Lawson ND, Weinstein BM. 2002. In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 248:307–318.

Li J, Zhang YP, Kirsner RS. 2003. Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix. Microsc Res Tech 60:107–114.

Manni L, Burighel P. 2006. Common and divergent pathways in alternative developmental processes of ascidians. Bioessays 28:902–912.

Manni L, Lane NJ, Zaniolo G, Burighel P. 2002. Cell reorganisation during epithelial fusion and perforation: the case of ascidian branchial fissures. Dev Dyn 224: 303–313.

Manni L, Zaniolo G, Cima F, Burighel P, Ballarin L. 2006. *Botryllus schlosseri*: a model ascidian for the study of asexual reproduction. Dev Dyn (in press).

Marti HH. 2005. Angiogenesis - a self-adapting principle in hypoxia. In: Clauss M, Breier G, editors. Mechanisms of angiogenesis. Basel, Switzerland: Birkhauser Verlag. p 163–180.

Martinucci GB, Dallai R, Burighel P, Lane NJ. 1988. Different functions of tight junctions in the ascidian branchial basket. Tissue Cell 20:119–132.

Matthysse AG, Deschet K, Williams M, Marry M, White AR, Smith WC. 2004. A functional cellulose synthase from ascid-

ian epidermis. Proc Natl Acad Sci U S A 101:986–991.
Myat MM. 2005. Making tubes in the *Drosophila* embryo. Dev Dyn 232:617–632.
Nakashima K, Yamada L, Satou Y, Azuma J, Satoh N. 2004. The evolutionary origin of animal cellulose synthase. Dev Genes Evol 214:81–88.
Nanka O, Valasek P, Dvorakova M, Grim M. 2006. Experimental hypoxia and embryonic angiogenesis. Dev Dyn 235:723–733.
Parker KK, Brock AL, Brangwynne C, Mannix RJ, Wang N, Ostuni E, Geisse NA, Adams JC, Whitesides GM, Ingber DE. 2002. Directional control of lamellipodia extension by constraining cell shape and orienting cell tractional forces. FASEB J 16:1195–1204.
Patan S. 2000. Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. J Neurooncol 50:1–15.
Patricolo E, Ferrarella A. 1973. Histochemical and biochemical studies on the collagen of the tunica of *Ciona intestinalis*. Riv Biol 66:115–134.
Peres JM. 1948. Recherche sur la genese et la regeneration de la tunique chez *Ciona intestinalis* L. Bull Inst Ocean 936:1–12.
Poschl E, Schlotzer-Schrehardt U, Brachvogel B, Saito K, Ninomiya Y, Mayer U. 2004. Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. Development 131: 1619–1628.
Risau W. 1997. Mechanisms of angiogenesis. Nature 386:671–674.
Roberts DM, Kearney JB, Johnson JH, Rosenberg MP, Kumar R, Bautch VL. 2004. The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. Am J Pathol 164:1531–1535.
Roos G, Landberg G, Huff JP, Houghten R, Takasaki Y, Tan EM. 1993. Analysis of the epitopes of proliferating cell nuclear antigen recognized by monoclonal antibodies. Lab Invest 68:204–210.
Sabbadin A. 1955. Osservazioni sullo sviluppo, l'accrescimento e la riproduzione di *Botryllus schlosseri* (Pallas) in condizioni di laboratorio. Boll Zool 22:243–263.
Sabbadin A. 1960. Ulteriori osservazioni sull'allevamento e sulla biologia dei Botrilli in condizioni di laboratorio. Arch Oceanogr Limnol 12:97–107.
Sabbadin A. 1969. The compound ascidian *Botryllus schlosseri* in the field and in the laboratory. Pubbl Staz Zool Napoli 37:62–72.
Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.
Schubert M, Escriva H, Xavier-Neto J, Laudet V. 2006. Amphioxus and tunicates as evolutionary model systems. Trends Ecol Evol 21:277.
Seipel K, Eberhardt M, Muller P, Pescia E, Yanze N, Schmid V. 2004. Homologs of vascular endothelial growth factor and receptor, VEGF and VEGFR, in the jellyfish *Podocoryne carnea*. Dev Dyn 231: 303–312.
Suchting S, Bicknell R, Eichmann A. 2006. Neuronal clues to vascular guidance. Exp Cell Res 312:668–675.
Sund M, Xie L, Kalluri R. 2004. The contribution of vascular basement membranes and extracellular matrix to the mechanics of tumor angiogenesis. Apmis 112:450–462.
Tammela T, Enholm B, Alitalo K, Paavonen K. 2005. The biology of vascular endothelial growth factors. Cardiovasc Res 65:550–563.
Tettamanti G, Grimaldi A, Valvassori R, Rinaldi L, de Eguileor M. 2003. Vascular endothelial growth factor is involved in neoangiogenesis in *Hirudo medicinalis* (Annelida, Hirudinea). Cytokine 22:168–179.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882.
Tiozzo S, Ballarin L, Burighel P, Zaniolo G. 2006. Programmed cell death in vegetative development: apoptosis during the colonial life cycle of the ascidian *Botryllus schlosseri*. Tissue Cell 38:193–201.
Tuszynski JA, Carpenter EJ, Huzil JT, Malinski W, Luchko T, Luduena RF. 2006. The evolution of the structure of tubulin and its potential consequences for the role and function of microtubules in cells and embryos. Int J Dev Biol 50: 341–358.
Vizzini A, Arizza V, Cervello M, Cammarata M, Gambino R, Parrinello N. 2002. Cloning and expression of a type IX-like collagen in tissues of the ascidian *Ciona intestinalis*. Biochim Biophys Acta 1577: 38–44.
Warburton D, Bellusci S, De Langhe S, Del Moral PM, Fleury V, Mailleux A, Tefft D, Unbekandt M, Wang K, Shi W. 2005. Molecular mechanisms of early lung specification and branching morphogenesis. Pediatr Res 57:26R–37R.
Waseem NH, Lane DP. 1990. Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. J Cell Sci 96(Pt 1):121–129.
Zachary I. 2005. Signal transduction in angiogenesis. In: Clauss M, Breier G, editors. Mechanisms of angiogenesis. Basel, Switzerland: Birkhauser Verlag. p 267–300.
Zaniolo G. 1981. Histology of the ascidian *Botryllus schlosseri* tunic: in particular, the test cells. Boll Zool 48:169–178.