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Whole-body protochordate regeneration from totipotent blood cells

(blastogenesis/*Botrylloides*/teratomas/Tunicata/vascular budding)

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ABSTRACT **Cell differentiation, tissue formation, and organogenesis are fundamental patterns during the development of multicellular animals from the dividing cells of fertilized eggs. Hence, the complete morphogenesis of any developing organism of the animal kingdom is based on a complex series of interactions that is always associated with the development of a blastula, a one-layered hollow sphere. Here we document an alternative pathway of differentiation, organogenesis, and morphogenesis occurring in an adult protochordate colonial organism. In this system, any minute fragment of peripheral blood vessel containing a limited number of blood cells isolated from *Botrylloides*, a colonial sea squirt, has the potential to give rise to a fully functional organism possessing all three embryonic layers. Regeneration probably results from a small number of totipotent stem cells circulating in the blood system. The developmental process starts from disorganized, chaotic masses of blood cells. At first an opaque cell mass is formed. Through intensive cell divisions, a hollow, blastula-like structure results, which may produce a whole organism within a short period of a week. This regenerative power of the protochordates may be compared with some of the characteristics associated with the formation of mammalian embryonal carcinomous bodies. It may also serve as an *in vivo* model system for studying morphogenesis and differentiation by shedding more light on the controversy of the "stem cell" vs. the "dedifferentiation" theories of regeneration and pattern formation.**

The Atlantic protochordate *Botrylloides leachi* is a very common encrusting colonial sea squirt, probably a Mediterranean species that has spread ubiquitously (1). Animals are found in very shallow water under stones and on algae, pilings, floats, and other substrata. Each colony is composed of few to thousands of genetically identical modules (zooids), each one 2–3 mm in length, which are embedded within a gelatinous organic matrix, the tunic. Zooids are arranged in systems of two parallel elongate and often serpentine rows with a long common cloacal cavity between them. All zooids within a colony are connected to each other via a network of blood vessels, from which pear-shaped vascular termini (ampullae) extend toward the colony margins. Light microscopy observations revealed that the blood vessels and the ampullae are very delicate structures with walls essentially one cell thick (2).

A colony originates from a sexually produced planktonic tadpole larva, which attaches to the substrate shortly after release, resorbs its tail, and metamorphoses to a founder individual, the oozooid. Colonies develop through a typical process of blastogenesis (asexual budding) from the thoracic body wall of the zooids, a phenomenon termed palleal budding (3). Palleal budding is a cyclic phenomenon, which terminates within 5–8 days. Buds (usually two per adult zooid) remain connected with the parent until fully developed. Then all parental zooids in a specific colony are synchronously resorbed, while buds mature to the zooid stage. This last event in the blastogenic cycle (termed takeover) is characterized by massive phagocytosis and is completed within 24 h from the first sign of zooid's degeneration. Budding in tunicates is believed to be a characteristic originally inherited by the ascidians from their remotest ancestry, though it has since been lost by some of them (4).

In addition to the normal process of palleal budding, the Japanese species *Botryllus primigenus* also exhibits a different pattern of budding, called vascular budding (5). This is possible only at a certain phase in the developmental cycle of the colony, where buds and zooids are developed from aggregations of blood cells at the bases of ampullae lying in the most vigorously growing edges of the colony. Unlike that species, another Japanese ascidian, *Botrylloides violaceum*, retains the capacity for vascular budding, but only along the walls of old blood vessels. More importantly, vascular budding in this species was never seen under normal conditions; it occurred only when a small piece of a colony deprived of zooids was isolated (6). In *Botryllus* from Naples, on the other hand, an isolated piece devoid of zooids (as an intact colony) never regenerated into a colony (or showed vascular budding), and none of the ampullae in an isolated piece showed the least tendency toward budding (7).

MATERIALS AND METHODS

Animals. Studies on invertebrate communities of Mediterranean hard bottom substrates have identified the existence of only two species of botryllid ascidians, *Botryllus schlosseri* and *Botrylloides leachi*. However, an inventory of ascidians collected along the Mediterranean coast of Israel (8) has noted, without giving any detailed description, the existence of *Metrocarpa* (= *Botrylloides*) *nigrum*, known from the Gulf of Suez, instead of *Botrylloides leachi*. This raises the question of which *Botrylloides* species is inhabiting intertidal habitats along the Israeli coast. A 2-year study on *Botrylloides* populations in Akko Bay (9, 10) revealed the existence of three distinct subpopulations, which differ in their color morphs, system organization, zooid orientation, relative abundance, reproductive seasons, allogenic responses, regeneration capacities, and zooid regression patterns. This study raises the possibility of the existence of three different species of putative varieties of one species in the area. Samples of the subpopulations (10) have not yet been formally determined taxonomically. Here we concentrate on colonies of only subpopulation 1 (9, 10). It is probably *Botrylloides leachi*, but it will be termed here merely as *Botrylloides* sp. to alleviate future confusion in the literature. *Botrylloides* colonies were collected from underneath stones and boulders, in shallow water along the Mediterranean coast of Israel. *Botrylloides* colonies grow naturally as large masses composed of up to several thousand zooids. Colonies were

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Abbreviation: EC, embryonal carcinoma.

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carefully peeled off stones together with attached thin layers of stony materials by using industrial razor blades and tied with fine thread to 5 × 7.5 cm glass slides, one colony or colony fragment per slide.

Experimental Procedures. Slides were held in the laboratory in a standing seawater system. Within 24 h of collection, steady ampullar contractions and expansions commonly resulted in vectorial gliding of whole or partial colonies from their natural calcareous substrate onto the glass slides. The translocated colonies were transferred to other glass slides and used in experiments after growth periods of weeks and months. Excision of zooids was performed under a dissecting microscope using fine metal needles and forceps. Zooids and their palleal buds were removed by tearing off the uppermost layer of the tunic and then carefully teasing out the zooids with their attached buds with the least possible damage to the extrazooidal blood system of the colony, which permeates the tunic. Subclones were achieved by cutting the colony with razor blades. Bleeding from injured blood vessels during subcloning or zooid excision stopped after only a few seconds.

RESULTS

Colonies of *Botrylloides* from the Mediterranean Sea have the ability to initiate vascular buds (9, 10). To further analyze this phenomenon, several experiments were conducted. Table 1 summarizes the results of three experiments: 23 colonies (with 5–55 zooids; Table 1, experiment a) from which all zooids were excised, 6 colonies that were divided into 2–5 subclones each before zooid excision (colonies with 6–71 zooids; Table 1, experiment b), and 15 oozooids (Table 1, experiment c) that were excised from their tunics. Except in one subclone that did not regenerate and died, all subclones and colonies survived and regenerated through the process of vascular budding. One to eight buds per colony or subclone were initially produced by this regeneration. Subsequently, only one bud in each subclone or colony matured to become a functional zooid, while all others disintegrated and were resorbed (Table 1, experiments a and b). In the oozooid experiment (Table 1, experiment c), 46.7% died without showing any sign of bud formation. In six out of eight cases in which regeneration was initiated through vascular budding (Table 1, experiment c), the bud developed further to the stage of a mature zooid.

Vascular buds developed in areas deprived of their zooids (such as an area in a subclone of a colony or within a whole colony that had had its zooids excised) as well as in almost any small isolated pieces of the vascular system. As a matter of fact, even a minute fragment of a blood vessel or an ampulla containing about 100–200 blood cells had the potential to regenerate into a fully functional zooid within 72 h from isolation. On the other hand, unharmed colonies or even pieces where at least one zooid was left intact did not regenerate through vascular budding (9).

In a second series of experiments, we isolated 28 small fragments of the marginal ampullae of a single colony, each containing 2–30 ampullae. All 28 isolated fragments started to regenerate within 1 week through the process of vascular budding. Some of them were histologically sectioned. All the others continued to regenerate (9), producing one zooid per fragment (Fig. 1). As in the previous experiment, vascular budding was initiated from an anarchic state where a mixture of blood cells was enclosed within a minimal size fragment of one blood vessel or several ampullae embedded in the tunic (Fig. 1*b*). Through a complex but well-regulated process, one or several buds appeared, of which only one per fragment matured subsequently to the state of a functional zooid (Fig. 1 *c–f*) containing all three primary germ layers. In all cases the zooids that regenerated through vascular budding continued to develop and formed new colonies through the process of palleal budding (9) (Fig. 1*f*). Vascular budding, or the progression from chaos to order may be summarized as follows: after a short period of blood flow restriction within the isolated fragment, a sluggish flow appeared where blood traveled for a short distance in one direction and then reversed to flow in the other. The source of this movement was located in the ampullae, which were seen to be slowly contracting rhythmically, driving the blood to and fro. When more than one ampulla was included within the isolated fragment, club-shaped branches of ampullae developed. Simultaneously, ampullae changed their orientation within the tunic matrix in a way that resulted in the establishment of a dense network of anastomosing blood vessels within a few hours to a few days (Fig. 1 *b* and *c*). Vascular buds were generated only within this new complex arrangement of meshed blood vessels, initiated by gatherings of blood cells within the lumen of the vessels. At first an opaque cell mass was observed (Fig. 2*a*). Through intensive cell divisions, a hollowed blastula-like structure resulted (Fig. 2), which thereafter cleared up and formed a bud that developed into a normal zooid.

Table 1. Vascular budding in whole colonies (experiment a), subclones (experiment b), and oozooids (experiment c) deprived of their zooids.

Exp.	Zooid no. before excision	Total no. of buds produced	Total no. of developed zooids
a	5	3	1
	6	1	1
	7	2	1
	7	3	1
	7	6	1
	10	1	1
	12	6	1
	15	1	1
	15	1	1
	15	5	1
	16	1	1
	17	4	1
	17	1	1
	18	1	1
	18	1	1
	20	1	1
	25	1	1
	30	1	1
	35	1	1
	39	2	1
	41	3	1
	53	4	1
	55	8	1
b	6	2 subclones, 5 buds	1 zooid/subclone
	19	5 subclones, 8 buds in 4, one subclone died	1 zooid/subclone
	28	2 subclones, 6 buds	1 zooid/subclone
	51	2 subclones, 4 buds	1 zooid/subclone
	61	4 subclones, 4 buds	1 zooid/subclone
	71	4 subclones, 4 buds	1 zooid/subclone
c	15*	8 animals, 8 buds	6†

Animals and subclones that failed to bud or started to bud but all their buds failed to develop to the zooid stage disintegrated and died.
*Oozooid number before excision.
†Total number of developed oozooids.

DISCUSSION

The tunicates (urochordates) and the vertebrates, being apparently of a common origin, are included in modern classifications in the same phylum, the Chordata. Therefore, the traits of tunicates may bear on the early history of the vertebrates. It seems plausible that the chordate stock at the

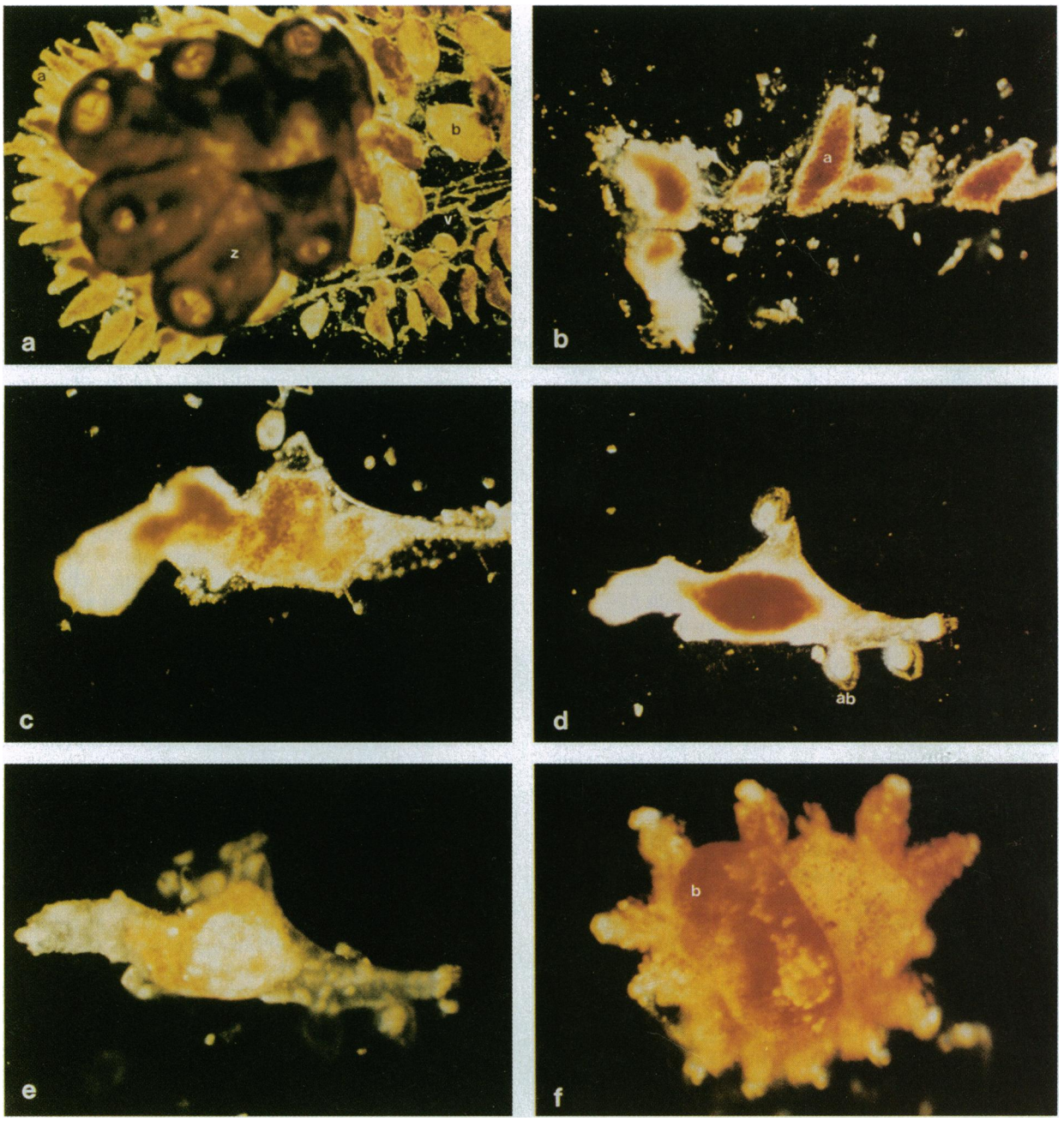

FIG. 1. Vascular budding in *Botrylloides*, the formation of functional zooids from a few minute fragments of blood vessels. (*a*) A *Botrylloides* colony growing in the laboratory on a glass slide. The zooids and buds of the colony are surrounded peripherally by ampullae, blind termini of blood vessels, containing a variety of types of blood cells. Each zooid is about 2 mm in length. (*b*) Immediately after removing the animal, leaving behind 6 minute fragments (0.2–0.8 mm in length). (*c*) Three days later, fusion between all fragments and the formation of a chaotic structure, which contains several hundred blood cells embedded within the tunic is observed. (*d*) After an additional week, an opaque mass of blood cells is formed in the middle of the regenerative structure. Three small "buds" of new ampullae are formed. (*e*) Four days later (2 weeks after sectioning), formation of an organized, still premature zooid is observed. (*f*) One week later, a complete functional zooid is observed. The next generation of zooid is developed through the normal process of bud formation and blastogenesis. a, Ampulla, a termini of a blood vessel; ab, ampullae bud; b, bud; v, blood vessel; z, zooid.

time of separation of the tunicate and the vertebrate lines of descendants still retained some potential for budding and colony formation (4). We presented here a regenerative power in a mature urochordate, probably originating from totipotent blood cells in the form of stem cells (11).

The ascidian tunicate's life cycle includes a motile larval form and a sessile adult stage. Development in these two stages differs fundamentally in regard to cell lineages and determination: while larval development is mosaic (isolated blastomers can develop only into the derivatives expected from the cell lineages), adult development or regeneration is regulatory (isolated parts may compensate and produce more than the derivatives expected) (this study and ref. 12). During the development of a chordate embryo, a single fertilized egg gives rise to about 200 different cell types (13) through highly controlled processes of cell commitments and differentiations. In a complete body regeneration of a colonial urochordate as described in this and earlier reports (5, 6), the transition from isolated totipotent stem cells to a fully regenerated organism also requires the generation of a considerable number of differentiated cell lineages. In the *Botrylloides* regeneration phenomenon, it occurs from a state of complete anarchy.

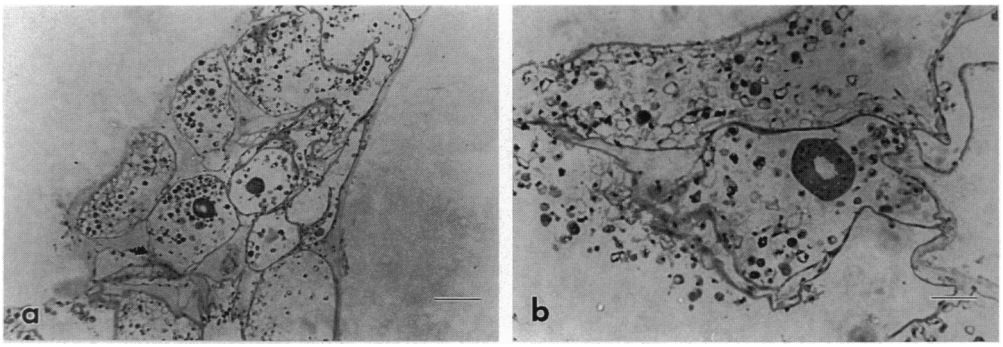

FIG. 2. Histological sections through early regenerative vascular buds within the lumen of ampullae, 2 days after isolating them from *Botrylloides* colonies. (*a*) Development of two spheres, one of which is hollow. (*b*) Development of one single-layered hollow sphere. (Scale bars: a, 60 μm; *b*, 30 μm.) Regenerating isolated ampullae of *Botrylloides* were removed from the glass substrates by razor blades. Ampullae were fixed in a 1% glutaraldehyde solution in seawater for 3 h, dehydrated by increasing concentrations of ethanol, and embedded in JB-4 plastic (Polysciences) for 24 h. Sections 2 μm thick were generated with a glass knife on a Reichert microtome. Staining was done by hematoxylin/eosin for histological observations.

While in many regenerating systems cells arise by dedifferentiation of functional tissue cells (14), recent studies have shown that populations of undifferentiated stem cells are the main source of regenerating organisms in platyhelminths (15) and cnidarians (16). These cells can live in the intact organism as quiescent cells, the "reserved cells" (17), or can function as stem cells, producing all types of differentiated cells (15). The regular budding processes of urochordates, such as blastogenesis in botryllid ascidians, represent an epithelic transdifferentiation system (18). On the other hand, whole-body regeneration from blood cells, as described here, most likely results from divisions of undifferentiated hemoblasts, the totipotent stem cells that freely circulate in the blood system (11). The stem cell model of development has also been proposed for tumor initiation and growth (19).

Although it is suggested here that the vascular budding of *Botrylloides* originates from blood cells alone, we cannot yet unequivocally rule out the possibility that other cell types, such as tunic cells and the epithelial cells of the blood vessels, also have a crucial role in this regenerative phenomenon. *In vitro* culturing of tunicate blood cells in 96-plate wells, with or without cells of the vessel wall, or *in vitro* culturing of whole small pieces of tunicate blood vessels did not regenerate to form a bud (C. Rabinowitz and B.R., unpublished results). Moreover, in *Perophora viridis*, a colonial ascidian that develops zooids from vascular stolons, a given dose of 5000 R of radiation may completely inhibit the budding process. However, injection of lymphocyte cells taken from an unirradiated part of the colony resulted in 55.6% of the budding and 11.1% of complete recovery from the lethal dose of irradiation, followed by a complete, normal growth pattern (20). This result, therefore, further emphasizes the significant, probably major role of blood-borne cells in vascular budding of colonial ascidians.

Besides its relevance to the evolution of development, totipotentiality and immortality of stem cells, this exceptional regenerative phenomenon of some urochordates also resembles some of the changes and processes associated with the mammalian teratocarcinoma, a bizarre neoplasm composed of foci of undifferentiated malignant cells interspersed within a chaotic array of somatic tissues (21). These tumors (best studied in the murine system), in which the embryonal carcinoma cells do not continue to multiply as undifferentiated cells, grow slower, usually ceasing growth within 6 weeks of initiation, and are not transplantable. Such tumors are known as teratomas, although this term is often used (as we use it here) to designate both the benign and malignant tumors (21–28). Teratomas represent tumors of pluripotential cells, either primordial germ cells or germ layers of early mammalian embryos (22). When arising in the ovary, teratomas are clearly the result of spontaneous parthenogenetic activation of an oocyte (22, 27). These tumors are characterized by the presence of a distinctive cell type known as embryonal carcinoma (EC) cells, which shows remarkable similarities to the cells of the early embryo and can differentiate into a wide range of cell types, representing derivatives of all three embryonic layers (22, 24). Such teratomas usually possess a wide variety of differentiated tissues, such as lens, bone, cartilage, teeth (both dentine and enamel), brain, glands, muscles, etc., which are haphazardly intermingled with the EC foci (28). Teratomas whose EC cells have all differentiated have stopped growing and are no longer malignant (22, 26).

Animals are the only kingdom of organisms developing from a blastula (29), a one-layered sphere of cells. Where there is no blastula, no animal is developed (29). It is therefore of great interest to note that the *Botrylloides* regenerative body (Fig. 2), the regenerative segment (≈300 epithelial cells) of the freshwater hydra that starts as a square flat sheet (30), and the EC foci all go through a stage that resembles a typical blastocyst in structure, a hollow sphere composed of a single pluripotent cell layer. Relevant also is the remarkable resemblance that was found between the cells that give rise to teratomas in mice and the cells that give rise to the developing mouse embryo. This resemblance is so close that in certain instances the tumor stem cells can join with their normal counterparts and develop into a completely normal mouse (21). However, it is clear that while the vascular budding of the tunicate represents a normal, successful response of regeneration/tissue renewal following damage, which also carries an ecological advantage (31), teratomas are merely a gross exaggeration of the process of tissue renewal (26) and embryological pathways (27) found in a variety of chordates including mammals (such as humans, mouse, horse, guinea pig), birds (fowl), fishes (guppy), and reptiles (lizard) (25). It should be noted, moreover, that while both the teratocarcinomas (21–28) and the urochordate bud regenerations develop in response to environmental perturbations, the buds are the device allowing survival of the organism, whereas the teratocarcinomas usually destroy the organism. The phenomenon of teratoma outgrowth may also be treated as a developmental accident or as a legitimate evolutionary puzzle. Moreover, the concept that teratomas are caricatures of normal embryonal processes (26, 27) may lead to a study of the selective evolutionary forces shaping this teratoma formation.

The EC cells (especially the best characterized F9 line) are widely used as a model system for studying differentiation in *in vitro* settings, as well as studying the intracellular events driving the commitment of cells to new phenotypes (13, 32). Similarly, the unique regeneration of botryllid ascidians may serve as an *in vivo* model system for studying not only

differentiation but also morphogenesis. The chordate teratomas may have had important functions in ancestral groups and may have survived to the present as nonadaptive evolutionary vestiges (26, 27). The vascular budding of some urochordates may, in addition to other uses, also serve as a tool for understanding the environmental causes for the spontaneously arising phenomenon of teratocarcinomas in the vertebrates.

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