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PAOLO BURIGHEL* – LUCIA MANNI*

Epigenetics in tunicates

Abstract - Tunicates are marine animals close to vertebrates, and have the ability, unique among chordates, to form large colonies of clonal individuals. Among tunicates, the most frequently studied ascidians represent an ideal model for investigating aspects of epigenetics evidenced during the various phases of the life-cycle. Interestingly, the typical mosaic development of eggs and embryos of ascidians, considered as a classic example of autonomous genetic control of genes for specification of cell lineage, may now be interpreted in the light of cytoplasmic and extracellular influences on the regulation of gene activity. In addition, the capacity of several species to create similar individuals by means of two different developmental pathways – embryogenesis and blastogenesis – and the high regulative power shown by colonies in natural or experimental modifications of the environment, reveal the importance of the epigenetic phenomena during the entire life cycle of these animals. Two species are of particular importance as models, the solitary *Ciona intestinalis* (whose genome has been recently sequenced) and the colonial *Botryllus schlosseri*. We report here examples of epigenetic phenomena in ascidians, to show that the various stages of the entire development of the phenotype are driven by a dynamic interplay of genome, cellular components and tissues, and their micro- and macro-environments.

Key word: Chordata, ascidians, asexual reproduction, environmental factors, cell differentiation, phenotype

INTRODUCTION

Monozygotic human twins are an obvious, elective “model” for studying aspects of epigenetics in complex organisms, i.e., the influence of environmental factors on identical genotypes. However, research is severely limited by the complexity and availability of the “model” and, of course, by ethics.

* Dipartimento di Biologia, Università di Padova, Via U. Bassi 58/B, 35131 Padova.
E-mail: paolo.burighel@unipd.it

Is there any natural availability of clonal, complex individuals as experimental models for epigenetic studies? Among chordates, to which man and the other vertebrates belong, the marine tunicates are a particularly interesting group of animals, because they are the closest relatives to vertebrates [Delsuc *et al.*, 2006] and because, unique among the chordates, they can reproduce asexually, giving rise to colonies formed of a large number of genetically identical individuals (blastozooids). The process of asexual reproduction, or blastogenesis, begins with the formation of buds which develop into zooids, and finds its counterpart in embryogenesis, i.e., the formation of individuals beginning from eggs. Despite the different starting point and the different environment in which the organs develop, the two processes lead to the formation of morphologically similar individuals. In addition, the great amount of information on various aspects of tunicate biology, including development, molecular biology, and phenotypic changes for overcoming unfavourable conditions, make these animals highly suitable for studying the influence of environmental factors in regulating gene expression in various contexts in time and space.

Among tunicates, the most frequently studied group, the ascidians, are sessile filter-feeding animals in the adult stage, but they also pass through an ephemeral planktonic stage as free-swimming tadpole larvae (Fig. 1). The larva, originating by embryogenesis, possesses a chordate body plan: the tail contains a central notochord and a dorsal nerve tube, both flanked by striated, bilaterally segmented mus-

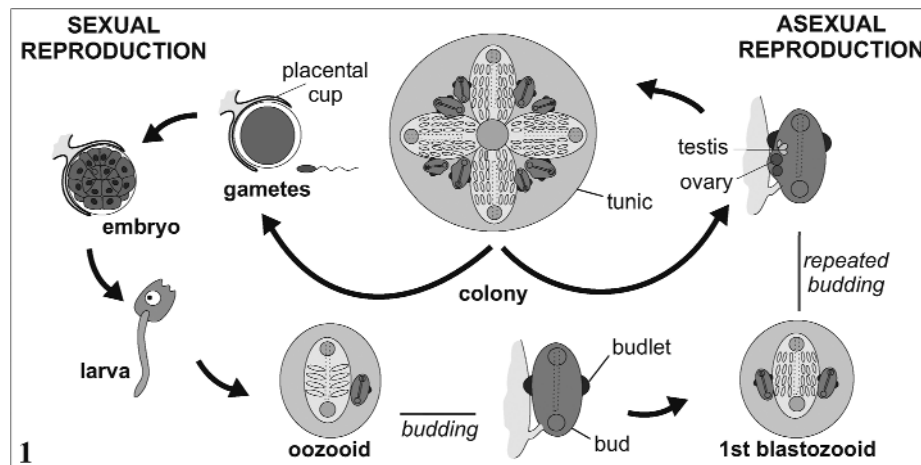


Fig. 1. *Botryllus schlosseri*. The life cycle includes a phase of sexual reproduction, giving rise to a free swimming larva, which metamorphoses into an oozoid. This is the founder of the colony which, during the phase of asexual reproduction, increases by budding a number of clonal zooids and developing gonads. Oozoids and blastozooids are shown in dorsal view. (Modified from Manni and Burighel, 2006).

culature [Burighel and Cloney, 1977]. In the solitary species *Ciona intestinalis* (whose genome has been recently sequenced [Dehal *et al.*, 2002]), the tiny central nervous system contains about 330 cells, thus providing a chordate nervous system in miniature, valuable for understanding structural elements and the developmental and possibly evolutionary processes of the vertebrate nervous system [Meinertzhagen *et al.*, 2004]. The larva also contains sensory structure (ocellus, statocyte, papillae) for swimming and choosing a substrate on which to settle. The larva then undergoes dramatic metamorphic changes, reabsorbing its tail, losing its chordate characteristics, and developing into a sessile invertebrate form, which is maintained throughout its remaining life-span.

In the case of colonial forms, oozoids are the founders of the new colonies, which grow by producing buds developing into adult zooids. Colonial ascidians offer the possibility of studying the mechanism regulating the interrelationships between each clonal individual and the colony as a whole, according to various environmental factors. They also allow us to study how developmental events are integrated to generate the animal form, since similar individuals can develop (oozoids from eggs, blastozooids from pluripotent somatic cells) by means of very different reproductive processes, i.e., embryogenesis and blastogenesis.

A) EPIGENETICS IN EMBRYO

Mosaic development

Starting from the classic work of Conklin [1905], ascidians were considered the best example of mosaic development, in that each blastomere seemed to follow a determined pattern of cell specification, irrespective of the experimentally modified environment in which it was left to develop. So, in normal conditions, the developmental fate of each blastomere within the cell lineage could be established in detail, and indicated that blastomeres development was under completely autonomous genetic control, and that a rigorous sequence of genes was expressed in each blastomere according to the different cell lineage. However, several papers [Reverberi and Minganti, 1946; Lemaire *et al.*, 2002; Nishida, 2005] have revealed that, also in ascidian embryos, the inductive processes occur between and influence contiguous tissues. It is now well established that, in ascidians, the expression/repression of specific genes responsible for the fate of each blastomere is not completely autonomous, but depends primarily on the presence of maternal factors, the determinants (including mRNAs). In *Ciona intestinalis*, these factors are translocated to specific regions by cytoplasmic and cortical reorganisation after fertilisation, and their positioning leads to the subsequent cell interactions and zygotic gene expression responsible for cell fate specification [Imai *et al.*, 2006, for ref.]. Thus, the ascidian mosaic model which, at first glance, seemed to be the best evidence that organ differentiation and phenotype depend only on the autonomous

achievement of a genetic program, is now becoming an elegant example of epigenetics: in the same embryo, cell lineage results from the continuous interrelationships of cytoplasmic factors and extracellular signals with the genome.

B) EPIGENETICS IN BOTRYLLID COLONIES

The Botryllus model

Among colonial ascidians, much work has recently focused on botryllids, in particular, on the cosmopolitan species *Botryllus schlosseri* (Fig. 2) which has become a key model for several studies, thanks to the possibility of rearing it in the laboratory and following its entire life-cycle [Sabbadin, 1955; Manni *et al.*, 2007]. Typically, colonies are composed of a few to several thousands of morphologically and genetically identical blastozooids, each interconnected via a colonial blood system. Zooids and blood vessels are encased within a translucent tunic. The eggs produced by blastozooids are internally fertilised and develop in the atrial chamber, giving rise to swimming larvae which escape from the parent and metamorphose into sessile oozoids. During embryogenesis, one or two buds are produced by the embryo and develop on the sides of the oozoid, representing the first blastogenetic generation of the colony. At a specific developmental stage, the buds produce budlets and, after several blastogenetic generations, the blastozooids produce mature gonads. Commonly, three generations co-exist in the colony; filtering adult zooids, their buds, and budlets (Fig. 2). The development of the three generations is synchronised: every week, the adults regress, are replaced in filtering activity by their buds, and a new budlet generation is produced. The new blastozooids, formed by pallial budding, inherit the parental epidermis, whereas their internal

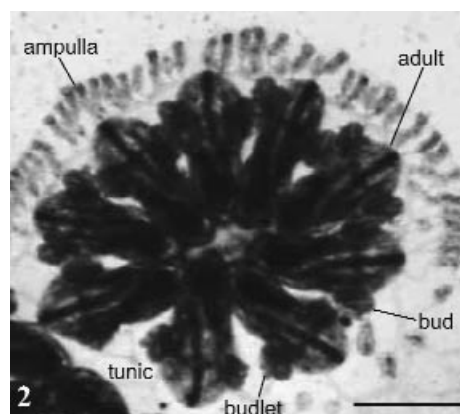


Fig. 2. *Botryllus schlosseri*: Ventral view of a colony seen *in vivo* to show three typical blastogenetic generations: adult, bud, and budlets. Scale bar = 1.4mm.

tissues are formed from pluripotent somatic cells in specific domains of the parent atrial epithelium. In botryllid ascidians, buds may also form from blood cells (vascular budding), at the base of a vascular ampulla or along a blood vessel. Buds consist of an outer vesicle, derived from the vascular wall, and an inner vesicle, originating from an aggregation of blood cells. Hemoblasts are the cells responsible for bud formation.

Aestivation/hibernation and vascular budding

Several botryllid species (e.g., *Botrylloides leachi*) offer wonderful examples of the influence of environmental factors on the colony phenotype. In severe conditions caused by extreme low or high temperature, the colony enters hibernation or aestivation, respectively (Fig. 3). It stops feeding and undergoes progressive alterations in its characteristics, with changes in the normal synchronised development of the three blastogenetic generations [Brunetti, 1976; Burighel *et al.*, 1976]. Alteration consists mainly of the regression of all adult zooids; loss of coordination of development in buds; regression and reabsorption of buds, beginning with the most highly developed ones; and the production of a large population of undifferentiated early buds from blood cells. At the same time, in the colonial circulatory system, the peripheral ampullae and vessels increase in extension and fill with blood cells, particularly phagocytes maintained in circulation owing to the contractile activity of the vessel epithelia. Later, the blood circulation in the tunic stops or is greatly reduced. The colonies become a sort of homogeneous red carpet composed of tunic with vessels and ampullae full of blood cells. When, after winter or summer, the colonies encounter favourable conditions, they reactivate, increasing circulation and producing new buds, which then develop into adult zooids.

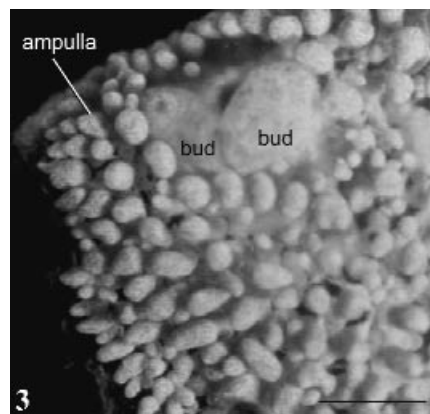


Fig. 3. *Botrylloides leachi*. Detail of a hibernating colony in early spring. Two buds begin to develop inside tunic, filled with ampullae and vessels. Scale bar = 0.3mm.

The capacity to restore the normal colonial phenotype (with the co-existence of the three blastogenetic generations), starting from buds forming from blood cells (vascular budding) may also be observed in *Botryllus schlosseri*. In this species, vascular budding does not usually occur, but can be caused experimentally by extirpating all the adults and buds. The remnant of the colony, composed only of tunic with its circulatory system, can survive for several weeks and maintain feeble circulation, thanks to the contractile ampullae and vessels. Here, bud primordia form by aggregation of blood cells, which are received into evaginations of the vessel epithelia. These buds grow and produce new buds by the normal pallial budding, finally becoming filtering adults.

It is noteworthy that, in both hibernating/aestivating colonies and vascular budding colonies, the normal co-existence of the three blastogenetic generations is re-established by means of developmental mechanisms differing from normal ones, because the first buds form starting from blood cells and not from the specialised staminal areas of the peribranchial epithelia (pallial buds). However, the co-existence of the three typical blastogenetic generations rapidly becomes re-established and the new adult zooids begin to filter. This phenomenon is an example of colony polymorphism under the influence of variations in environmental factors.

Regulation and plasticity of the colony

Botryllus schlosseri can overcome various unfavourable environmental conditions by modifying the normal developmental pattern of its blastogenetic generations.

In a series of elegant experiments, in 1958 Sabbadin analysed the extent of the regulative power and mechanisms used by colonies in order to respond to various environmental situations. Several biological problems were approached with various experimental techniques: extirpation of zooids and/or buds, or isolation and grafting of buds. Among other conclusions, results showed that: a) the co-existence of three blastogenetic generations can be altered, b) blastogenetic powers are bilateral but asymmetric, and vary according to the condition of the colony; c) the final size of buds depends on competition with other buds of the same zooid; d) ablation of buds modifies the duration of parental life; e) the achievement of sexual maturity is influenced by good environmental conditions. A main conclusion was that colonies represent physiological units in a dynamic, balanced situation based on competition between zooids of the same and co-existing generations: if environmental conditions worsen, sexual reproduction is arrested, the budding rate is reduced to one bud per zooid, and the colony stops growing. It is now of extreme interest to re-examine these aspects of epigenetic controls on organism development with the modern approaches afforded by molecular biology, and to identify and analyse the expression/repression of specific developmental genes involved in the regulation of the plasticity of the colony.

Polarity of bud axes and bilateral asymmetry

An interesting field of research is determination of the polar axes in developing organisms, with particular attention to the role of the developmental genes involved. In some cases, the mechanisms and genes responsible for specifying embryo axes have been established, showing that maternal factors play an important role in the process (in *Drosophila*, specification occurs mainly before fertilisation; in ascidians, it occurs after the cytoplasmic rearrangement of maternal factors after fertilisation). There is little information on the inductive and genetic mechanisms specifying axes in buds and on the possible role of parental tissues and the micro-environment in the process.

This topic was faced by Sabbadin in *Botryllus schlosseri*, who observed that, in special experimental conditions [Sabbadin, 1956; Sabbadin *et al.*, 1975], ablation of zooids causes buds to develop reverse bilateral asymmetry (*situs inversus viscerum and cordis*) (SIV), with zooids that, opposite to the normal situation, have their digestive tract located on the right and the heart on the left. The reversion also influences the blastogenetic and gonadogenetic powers of the zooids - more greatly developed on the left and right, respectively. Thus, Sabbadin was able to rear colonies with all zooids having SIV and showed that, although the reversed bilateral asymmetry of a zooid was transferred to those of the successive blastogenetic generations, it was not genetically inherited by their embryos. Moreover, in studies of vascular buds and grafting of buds between normal and SIV colonies after extirpation of zooids, he demonstrated that: a) both antero-posterior and dorso-ventral axes are not “intrinsically impressed” in early buds, but are imposed by environmental factors in such a way they can be restructured in early developmental phases by changes in external factors: polarisation of the antero-posterior axis is established by vascularisation, i.e., it coincides with the entry/exit points of vessels in the bud; b) instead, bilateral asymmetry is rigorously predetermined in the bud primordium and, in both normal and reversed conditions, always corresponds to that of the parental zooid. This is also true for buds produced by the colonial matrix by vascular budding, because these buds always develop with asymmetry corresponding to that of the removed zooids. Thus, this model offers the possibility of studying how genetic and epigenetic factors influence body axis formation. At present, in agreement with Sabbadin *et al.* [1975], we assume that, in the asexual reproduction of *Botryllus schlosseri*, “the developmental pattern transmitted from parent to bud is highly regulative and not mosaic in form”.

Chimaeras and winner cells

When two colonies of *Botryllus schlosseri* moving on the substrate come into contact, they display their capacity for allorecognition by fusing their tunics and circulatory systems, or by rejection, producing a necrotic reaction in the contact

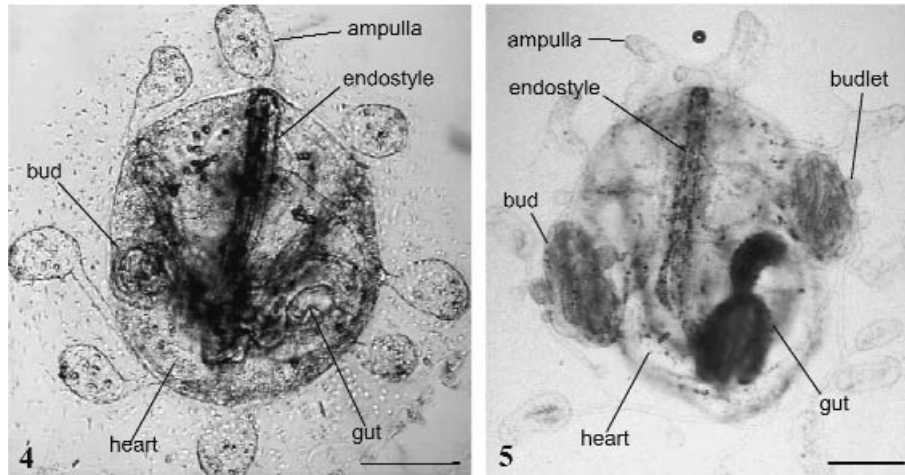
zone. Fusion or rejection is governed by a single highly polymorphic locus called the FuHC (fusion/histocompatibility) [Sabbadin, 1962; Scofield *et al.*, 1982] as follows: if the two colonies share one or both FuHC alleles, they will fuse; if they have no alleles in common, they will reject. Fusion of colonies involves exchange of blood and migrating cells between the two colonies, with the formation of chimaeric colonies having cells with two different genotypes which compete with each other to create their phenotype in the new colonial environment. Evidence of “winner” and “loser” genotypes was first demonstrated in somatic and germ cells by Sabbadin and Zaniolo [1979], who analysed the phenotypes and collected the descendants of chimaeric colonies. Their results strongly suggested that toti- or pluripotent stem cells could be transferred into compatible colonies and differentiate in their new environmental conditions.

This problem has recently been investigated with modern techniques by labelling isolated cells and transplanting them into the circulation of compatible host colonies [Laird *et al.*, 2005]. The approach allows the formation of chimaeras and the identification of heterologous components, avoiding the need to fuse colonies and collect descendants. It is thus very suitable for studying the basis of cell differentiation in tunicates, following the cell commitment before transplantation and the influence of the various landing niches, and thus the various environmental factors, in directing final cell differentiation.

C) EPIGENETICS IN TWO DEVELOPMENTAL PATHWAYS

A remarkable aspect of *Botryllus schlosseri* is its capacity to form similar individuals through two different developmental processes, i.e., embryogenesis and blastogenesis (Figs. 4, 5). Since these two pathways start from completely different starting points (the egg, and a domain of somatic stem cells), it is of interest to examine the various developmental phases of the two differentiating organisms in order to understand the tissue and genetic mechanisms leading to the final morphology. The issue has aroused the interest of many authors, thanks to the possibility of verifying whether steps in embryogenesis are repeated during blastogenesis, or if the latter is a completely new form of development. Of interest also is if and when similar “cassettes” of critical developmental genes are activated in differing environmental situations to direct the formation of comparable structures [Tiozzo *et al.*, 2004].

In a series of studies, Manni and Burighel [2006 for ref.] compared the two pathways in detail, demonstrating that, as soon as the body pattern is established and the rudiments of the main organs are formed, several similarities may be observed between the two processes in *Botryllus schlosseri*. The stomodeum, developing neural complex, branchial basket with the ventral endostyle, and peribranchial/atrial chambers, are easily identified on morphological bases in both embryo and bud: this information provides us with a valuable morphological framework.



Figs. 4, 5. *Botryllus schlosseri*. Oozoid (Fig. 4) and blastozoid (Fig. 5) seen *in vivo*, ventral view. Although derived from different pathways (embryogenesis and blastogenesis, respectively), both show similar body morphology. Scale bar = 0.35 mm (Modified from Manni and Burighel, 2006).

Attention was paid in particular to the formation of the neural complex, which in ascidians is constituted of the cerebral ganglion, neural gland and dorsal strand. Results showed that a number of corresponding events could be recognised in oozoids and blastozoids [Burighel *et al.*, 1998; Manni *et al.*, 1999]. In both cases, the neural complex derives from a blind tubular structure – the neurohypophysial duct in the embryo, the dorsal tube in the bud – which derives from a localised, thickened epithelium – the anterior neural plate in the embryo, and the dorsal region of inner vesicle in the bud [Mackie and Burighel, 2005]. The two tubes have the same anatomical relationships with adjacent organs, being dorsal, and grow anteriorly to reach and open into the prospective oral siphon. In both cases, the cerebral ganglion is composed of neuroblasts, which delaminate from the wall of the tubular structures and migrate a short distance to reach their final position under the neural gland. On the basis that neural rudiments represent homologues of the hypophyseal and olfactory placodes of vertebrates [Manni and Burighel, 2006], we analysed the expression of the gene *Bs-Pitx*, a homologue of the panhypophyseal *Pitx* gene of vertebrates by *in situ* hybridization [Tiozzo *et al.*, 2004]. At first, unlike eggs, *Bs-Pitx* is expressed in the entire early bud but, when organogenesis occurs, it is expressed in comparable prospective regions of embryos and buds and labels the same territories in the two developmental pathways (the rudiment of the neural gland aperture, some anterior-ventral neurons of the forming cerebral ganglion, a ring of tissue round the mouth). Data indicate that the genetic pathways regulating placode formation in embryo are co-opted for blastogenesis and become

activated by the corresponding cellular micro-environment. As a result, starting from different points (mosaic embryo and pluripotent somatic cells), embryonic and blastogenetic development leads to the formation of comparable territories in which cell interactions activate the same genes, and their interplay with environmental factors produces the same final phenotype in both cases.

CONCLUSIONS

Tunicates, and particularly the colonial ascidian *Botryllus schlosseri*, represent a useful model for studying, during their entire life-cycle, aspects of epigenetics according to the original view of Waddington that genetic control is not autonomous, but responsive to environmental signals, i.e., factors internal to organisms or external to them. All the examined cases show that the dynamic interplay of genetic components with cellular and extracellular environments is responsible for the phenotype of each component and of the entire organism, in each stage of its development and in its final, mature form.

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