

## Report of Meeting

**II scientific meeting of the Italian Ascidiologists, 30 June – 1 July 2008, Department of Animal Biology, University of Palermo, Palermo, Italy**Organizers: **N Parrinello, V Arizza, M Cammarata, M Vazzana, A Vizzini***Marine Immunobiology Laboratory, Department of Animal Biology, University of Palermo, Palermo, Italy***Session 1. Development and morphogenesis****Sensory organs in the oral siphon of Pyrosoma****P Burighel, L Manni F Caicci***Department of Biology, University of Padua, Padua, Italy*

In previous papers we studied the sensory structures in the branchial siphons of ascidians evidencing the presence of sparse primary sensory cells and secondary sensory cells. The latter constitute an extended mechanosensory organ (the coronal organ) presents in the oral siphon of all the examined ascidians. For position, origin and ultrastructural features these sensory cells have several correspondences with the hair cells of vertebrates. Thus, the possibility that they derive from embryonic territories sharing common aspects to the neural placodes of vertebrates was proposed.

In order to verify whether the secondary sensory cells can be considered a common feature of all tunicates, we studied a representative of the Pyrosomatida (Thaliacea). Pyrosomes are planktonic free-swimming tunicates forming tubular colonies. The individuals are embedded in the wall of the tube, with their oral siphon opened at the outer surface and their cloacal siphon opened into the common, central cavity. A water current passes each individual from the oral to the cloacal siphon. The oral siphon is rich in musculature and is covered by the epidermis also in its interior surface. A crown of tentacles run at the base of the siphon; in particular, a long ventral tentacle extends down toward the branchial cavity. With series of thick (1  $\mu\text{m}$ ) and thin sections we have evidenced that two kind of sensory cells are present in the oral siphon of pyrosomes. One is represented by the sensory cells of small, scattered cupular organs, which are formed of the central sensory cell accompanied by two supporting cells. The sensory cell is covered by a tunic-like cupula and its apical region has an extracellular canal containing a long cilium emerging among numerous long microvilli. The organisation of these cupular organs strictly recall the mechanoreceptor capsular organs of some ascidians. The second kind is represented by secondary mechanosensory, ciliated cells whose base contact neurites directed to (or arriving from) the brain. These

cells run in a continuous row along the border of all the tentacles, forming a coronal organ corresponding to that of ascidians and resemble vertebrate hair cells for the presence of one-two cilia surrounded by a dozen of microvilli. The data suggest that the coronal organ is a common feature of tunicates.

**Differentiation of sensory papillae in the embryo and larva of *Botryllus schlosseri*****F Caicci, P Burighel, L Manni***Department of Biology, University of Padua, Padua, Italy*

In ascidians, the main class of the tunicates, the primary sensory cell constitutes the basic cellular element of most sensory structures, both in the free-swimming larva and in the sessile adult. The larva has sensory structures specialized for reception of gravitational, light, tactile and chemical stimuli. In particular, the sensory papillae are considered chemoreceptive structures, localized in rostral position of larval cephalenteron, which recognize and select the suitable substrate for adhesion at the onset of metamorphosis. By light and electron microscopy, we have studied the development and the structure of the sensory papillae in the embryo and larva of *Botryllus schlosseri*. In this species, the papillae are three in a triradial arrangement and were defined in the past as "ganglionated type" for the presence of a ganglion to their base.

The first rudiments of the papillae appear in early embryo in form of three distinct ectoderm evaginations, delimiting a small cavity in communication with the underlying mesenchymal space. In the successive stages, the cavity of each papilla enlarges to receive the papillary ganglion, which is constituted of primary sensory cells. These latter are bipolar neurons possessing receptor terminations in the tunic and a long axon extended to the central visceral ganglion. The papillary ganglion becomes recognizable under the epidermis owing to extension of the sensory neuron bodies into the papillary cavity.

In the swimming larvae, the papillae are formed of three cell types: central sensory cells of papillary ganglion, peripheral sensory cells and cubical parietal cells. Immediately before the adhesion to substrate, the papillae modify their shape: they elongate, the

ganglion becomes embedded in the papillary ectoderm, and the receptor terminations, crossing the tunic layer, are exposed to external side through fissures of the tunic. After adhesion, the papillae are retracted, while the central interpapillary area reaches the substrate; at the same time, the larva protrudes the blood ampullae for the definitive attach to the substrate.

### **Developmental expression and gene organization of protochordate synapsins: highlights on basic functions**

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Synapsins belong to a family of neuron-specific phosphoproteins and are involved in several functions correlated with neurotransmitter release and synaptogenesis (reviewed in Hilfiker *et al.* Phil.Trans. R. Soc. Lond. B 354: 269-279, 1999). The comprehension of the basal role of the synapsin family is hampered in vertebrates by the presence of multiple members. Then, the study of homologous genes in protochordates, such as ascidians and amphioxus, lacking the whole genome duplications characteristic of vertebrates, could help to better understand the complex functions of synapsins. Therefore, we have cloned and analyzed two synapsin genes, *Ci-Syn* in the ascidian *Ciona intestinalis* and *amphisyn* in the amphioxus *Branchiostoma floridae*. Our results show the presence of a single synapsin gene in both organisms, and the occurrence of a second transcript by alternative splicing in *B. floridae*. Our genomic analysis reveals an high homology degree with vertebrate synapsins, in particular with synapsin III, the occurrence of three conserved domains A, C and E, and the presence of the classical nested organization of a TIMP (tissue inhibitor of metalloproteinase) gene in the intronic sequence. Moreover, we have localized by in situ hybridization *Ci-Syn* and *amphisyn* transcripts exclusively at neuronal levels and in particular in a relevant portion of developing neurons, suggesting that the basic role of synapsins as regulators of neurotransmission and synaptogenesis has been conserved during evolution.

### **Sex hormone profiles in *Ciona intestinalis* (Ascidacea Urochordata) and their modulation by Tributyltin (TBT)**

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Regardless of species, steroid hormones are derived from cholesterol as a common precursor. In chordate, genome sequencing showed that cytochrome P450 (CYP) enzyme system play integral

roles steroidogenic pathways and these processes are well conserved in many species. Interestingly, in the protochordate *Ciona intestinalis*, several rate-limiting steps involving CYP enzymes are lacking, although steroid hormones have been detected. The aim of this work is investigate the synthetic pathway of steroid hormones in *C. intestinalis*. Given the lack of integral CYP enzyme genes, our hypothesis is that steroid hormone synthesis in *C. intestinalis* are probably mediated through an alternative pathway involving second messenger (camp) that activate protein kinases to regulate acute steroid production at level of substrate (cholesterol) availability. Our preliminary data showed an increase in cholesterol and Ca<sup>2+</sup> concentrations in ovary of *C. intestinalis* after *in vitro* TBT treatment. Based on these results, we started the investigation of steroid hormones levels in *C. intestinalis* after *in vitro* TBT treatment. Analysis on testosterone, 17 $\beta$ -estradiol, aldosterone, progesterone and cortisol concentrations has shown appreciable variations in these hormones. Thus, we are currently evaluating whether these responses could be activated or enhanced in the presence of camp or second messenger activator (forskolin).

### **Ion current and molecules involved in meiotic progression and fertilization of *Ciona intestinalis* oocytes**

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We performed an electrophysiological characterization of immature oocytes of the *Ciona intestinalis* at different stages of growth and meiotic competence.

Oocytes showing a clear germinal vesicle (GV) have been classified on the basis of diameter and cytoplasm pigmentation as follows: stage A: <70 $\mu$ m diameter with a clear cytoplasm; stage B: 70-120 $\mu$ m diameter with a yellow cytoplasm; stage C: >120 $\mu$ m diameter with a brown cytoplasm.

Using the whole-cell voltage clamp technique on the oocytes collected from the ovary and deprived of accessory membranes, we showed for the first time a high L-type calcium currents activity at the stage A, these currents significantly decreased through meiosis up to the metaphase I (MI). Oocytes at stage B showed the first appearance of sodium current activity that are still active at the MI phase. In vitro maturation experiments in calcium free sea water showed a significant reduction of maturation of oocytes at the stage C.

Intracellular calcium release was higher in MI than in previous stages; at last by using cyclic AMP activators we showed a significant decrease of the germinal vesicle breakdown.

Fertilization current generated in sodium free sea water was significantly lower than the control, furthermore, oocytes fertilized in absence of sodium showed high development of the anomalous "rosette" embryos.

Taken together these results imply: i) an involvement of external calcium in modulating oocytes growth, meiotic competence acquisition, prophase/metaphase transition and calcium stores refilling necessary for oocyte contraction at fertilization ii) a role of cyclicamp in maintaining the meiotic arrest as it happens in mammals; iii) a role of Na<sup>+</sup> currents during electrical events at fertilization and subsequent embryo development.

#### **A morpho-functional analysis of the adhesive papillae of ascidian larvae**

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Most ascidian larvae adhere to the substrate by mucous secreted by the adhesive papillae, which in nearly all species are made of elongated secretory cells and primary sensory neurons. The exceptions are the papillae of the species of the genus *Botrylloides*, which are considered to have only a sensorial function (Grave, 1934) and that of genus *Clavelina*, which are reported to be only secretory (Turon, 1991). We analysed the adhesive papillae of *Botrylloides leachi*, *Clavelina lepadiformis* and *Diplosoma listerianum* by histological analysis and by immunolocalization of serotonin and  $\beta$ -tubulin. We demonstrated that in all analysed species the adhesive papillae have both sensorial and secretory function. In particular, larvae of *B. leachi* have a lot of secretory cells gathered in the centre of a small area between the three adhesive papillae, forming a glandular organ. This organ could allow the adhesion of the larva to the substrate also by a sucker mechanism. The adhesive papillae of *C. lepadiformis* contain, in addition to the secretory cells, many primary neurons, the axons of which gathered to form papillary nerves.

All analysed species contain two different types of neurons with different localisation and possibly a different function. The first type neurons, likely mechanoreceptors or chemoceptors, emerge from the tunic at the apex of the papillae and play a role in the substrate choice. The second type neurons are localised in more lateral position in the papillae and do not contact the substrate until the papillae are retracting owing to a permanent adhesion. These neurons contain serotonin and they could play a role in triggering metamorphosis. On the basis of the role of neurotransmitters in ascidian metamorphosis, we hypothesize that serotonin released by the peripheral neurons after the permanent adhesion may acts as an internal signal triggering the metamorphosis process.

#### **The adult musculature in *Botryllus schlosseri*: differentiation and gene expression**

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Typically, ascidians have three types of muscles: skeletal in the larva, cardiac and smooth in the post-metamorphic sessile organism. The larval and cardiac muscles are striated, with vertebrate-like arrangement

of myofilaments. Instead, the smooth body-wall musculature has intermediate characters between smooth and striated muscle of vertebrates. We studied the musculature in the blastozooids of *Botryllus schlosseri* analysing its organisation, differentiation and gene expression. We isolated and characterised two transcripts resulted homologous to muscle genes of other adult ascidians: a muscle-type actin (*bsma2*) and troponin T (*bstnt-a*); moreover, we obtained also the genomic sequence coding for *bsma2*.

Phylogenetic analyses showed a close relationship between urochordates and vertebrates muscle genes. The *bsma2* genomic sequence was compared in the exon-intron organization with other muscle and cytoplasmic-type actin genes of both invertebrates and vertebrates. Our analysis showed that intron positions are conserved in ascidian and in the other deuterostomes.

We detected the expression of the two genes by *in situ* hybridization (ISH), in order to follow the muscle development throughout the blastogenetic cycle of *B. schlosseri*. The ISH, in parallel with phalloidin staining experiments, showed that the first diffuse signal of muscle differentiation appears in the intersiphonal epidermis of young buds. Then, the muscle fibers differentiate into the body-wall, while an intense expression of *bsma2* marks the heart myocardium just when it begins contractions. The ultrastructure of smooth muscle cells was also investigated during differentiation from mesenchymal cells in the bud, in the adult, and in the fibers contraction during regression of zooids.

#### **Preliminary data on antiproliferative effects in haemocyte extracts of the ascidian *Styela plicata* (Stolidobranchiata, Styelidae)**

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Several marine invertebrate are among the most important sources of biomedically relevant natural products. A recent expansion of the studies of marine natural products involves the search for compounds with antitumor properties. In particular, among the compounds that have been isolated from sponges, bryozoans, and tunicates, several have been widely described (Discodermolide, bryostatin-1, ET-743), and have progressed to pre-clinical or clinical-trial phases. Tunicates, organisms having a wide geographic distribution, have increasingly become the target of natural products research and have been reported to harbour metabolites with a wide range of biological activities such as metal accumulation, sclerotization of their extracellular matrices, and antimicrobial activity. Some of these activities in marine invertebrate are also carried out by the circulating hemolymph that presents different types of haemocytes embedded in the liquid plasma and contains biologically active substances that contribute to the innate immune mechanisms. Recent researches have shown that the extracts of the total haemocytes population from different ascidians

contain several compounds with an antimicrobial activity such as antibacterial, antifungal and antiviral ability.

The aims of the present study was to extend the knowledge on the biological activities of these compounds testing the antiproliferative potential of the haemocyte extracts from the solitary ascidian *St. plicata* on leukaemia cell lines.

### **Comparative ultrastructural investigation on the adhesive papillae of the swimming larvae of three Ascidiidae species**

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Ascidian swimming larvae bear three peculiar organs of ectodermic origin, named “palps” or adhesive papillae, located in the anterior region of cephalenteron. Term “adhesive” is correlated to one of the function of these structure based on secretion of an adhesive substance which enables swimming larvae to adhere to a substratum. Recently a sensory function has also been described in some Phlebobranchia papillae with a simple morpho-functional organization. There are few ultrastructural investigations in literature, sometimes disputed, able to make clear papillae cells functions. To clarify this problem, a comparative investigation has been carried out, just in this work, about ultrastructural and morpho-functional organizations of adhesive papillae of three ascidian swimming larvae, *Ascidia malaca*, *Phallusia mammillata* and *Ciona intestinalis*. The investigations has been carried out by transmission electronic microscopy and confocal microscopy. Papillae of above-mentioned ascidian swimming larvae, are three in number and they are located at the vertices of a triangular field. According to a recent classification scheme by Burighel and Cloney, they are coniform and non-eversible simple type papillae. In the apex of each papilla there is an hyaline cap with an electron-dense substance made of proteoglycan component. It's quite certain that this substance is used by ascidian larvae to adhere to the substratum. Ultrastructural investigations carried out on the adhesive papillae of *A. malaca* and *P. mammillata* have not emphasize significant ultrastructural differences in the papillae cells body. *A. malaca* and *P. mammillata* papillae are made of three types of cells that characterize their functions: a) colocytes, b) axial columnar cells, c) sensory cells. Colocytes, whose ultrastructure is typical of cells with secretory activity, are certainly deputed to form adhesive secretion. They lie in a median or semilateral side of the papillar body. Axial columnar cells that show an elongated shape, lie in the mid-central papillae region and they are characterized by the presence, in their apical part of long microvilli that run along the whole of hyaline cap length. Above a structural supporting role, it is supposed a possible sensory function concerning these cells. Sensory cells presents an ovoidal and elongated shape holding on tight at their base like a funnel lying a lateral and marginal position in papillar body. These

type of cells have been described in *A. malaca* which can be classified as primary sensory neurons. These are characterized by the presence of a single cilium in the apical end and by an axonic process at their base extending over papilla. Ultrastructural investigations on the adhesive papillae of *C. intestinalis* confirmed, even if showing some differences, ultrastructural likeness the two above-mentioned species of adhesive papillae. Colocytes with secretory activity, sensory cells and columnar axial cells come into sight also in *C. intestinalis* papillae. The columnar axial cells ultrastructure is different from the *A. malaca* and *P. mammillata* papillae. In the apical part of these cells there are not microvilli but digitiform protusions with numerous microtubules inside running paralleling along longitudinal cell axis. The ultrastructure of these cells suggest their lengthening ability to allow digitiform protrusions to put into contact with the cuticular layer of hyaline cap apex, during step before the adhesion. Ultrastructural characteristics suggest that they can also perform a sensorial duty but their mechanism is still unclear. The observation was carried out by confocal microscopy on the ascidian larvae just before or at the beginning of adhesion to the substratum (6-7 hours from hatching). The use of anti- $\beta$ -tubulin antibody, have shown an extensive nervous network in all three Ascidiidae species starting from the papillar base and converging into only one nerve extending up to the cerebral vesicle. These data confirm previous observations carried out with confocal microscopy on *A. malaca* and *P. mammillata*. Larvae. In *C. intestinalis* adhesive papillae marked with anti- $\beta$ -tubulin antibody, the fluorescence is more bright than *P. mammillata* and *A. malaca*. Probably this result is correlated with apical protrusion rich in microtubule. Ultrastructure observations of some neurons being in the anterior region of cephalenteron, extending from the base of papilla to the cerebral vesicle, have been emphasized in this work. These neurons are in the same position of the ones described like RTEN (rostral trunk epidermal neuron) by Imai and Meinertzhagen and they are formed by cellular ovoidal body extending into a long axon. Ultrastructural of these neurons have pointed out likeness with vertebrate neurons.

Concluding, ultrastructural investigations have emphasize only some difference between papillar cells of *A. malaca*, *P. mammillata* and *C. intestinalis* larvae, confirming adhesive and sensory functions.

### **Evolution of anterior Hox regulatory elements among Chordates**

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Hox genes determine AP identities from insects to

vertebrates in all the animal species where they have been studied. Their organization in cluster and spatio-temporal colinearity of expression have usually been considered their more significant characteristics. The sequencing of the genomes of even more species has helped to understand various aspects of Hox gene organization.

Among Chordates, particularly interesting are the anterior groups of Hox genes since their expression is coupled to the control of regional identity in the anterior nervous system, where the highest structural diversity is observed. Hox genes exert a fundamental role in vertebrate hindbrain formation and segmentation. Both ascidians and amphioxus lack a segmented hindbrain but at the same time show restricted expression patterns of anterior Hox genes like their vertebrate counterparts.

Changes in gene regulatory regions are considered a driving force for the evolution of more complex body plan structures. To investigate how Hox gene regulation changed and evolved in the chordate lineage, we have analysed and compared various Hox regulatory regions of three chordate species, amphioxus, ascidian *Ciona intestinalis* and mouse. In particular, we focused our attention on elements controlling anterior Hox genes expression and active in the anterior nervous system.

We have studied the ability of amphioxus and mouse regulatory elements to function in *Ciona* embryos and to reproduce the segmental expression pattern typical of Hox genes. Conversely, we have also analysed the ability of *Ciona* nervous specific enhancers to be recognized by the mouse regulatory mechanisms.

#### **Anatomy of *Ciona intestinalis*: larval, metamorphic and juvenile phases**

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The solitary ascidian *Ciona intestinalis* is a model organism frequently exploited for comparative investigations of chordate development and for unravelling the molecular mechanisms underlying morphogenesis and cell fate specification. This species has an indirect development comprising the embryonic phase, free swimming larval stage, metamorphosis to form a sessile juvenile and, ultimately, the adult with the mature reproductive organs. Some of the first developmental stages have been described in detail using different classical and modern techniques. Thank to these lines of study, cell lineages in ascidian embryo were thoroughly described from fertilized egg until the larval stage. Recently, also successive developmental stages, such as the metamorphosis and the juvenile differentiation, has begun to be investigated, although less extensively. All these studies concur to improve our knowledge on *Ciona* development, but several questions remain to be solved, because each technique has intrinsic advantages and limits. In this light, the authors discuss recent data from other laboratories examining also results obtaining recently by confocal microscopy. Despite this technique permits the three dimensional reconstruction of the main developmental events in young embryos, it remain unsatisfactory in resolving precise aspects of

interaction and differentiation between cells and/or tissues in successive stages. This is particularly relevant for late larvae and during metamorphosis, when the complex organogenesis of new rudiments and body reorganization occur accompanied by tissue shrinkage and disaggregation of embryonic structures,. The Authors exemplify, analysing by means accurate reconstruction of specimens with histology and ultrastructure, critical stages of *Ciona* development. The data show that also today the classical histology and the ultrastructure are complementary and essential techniques for the understanding of the anatomical organization of tissues, organs and the organism as a whole, and can represent fundamental reference for interpretations of sections in which tissues are partially labelled, as in *in situ*-hybridization or in immunocytochemistry.

#### **Distribution of neural phenotypes in the larva of *Ciona intestinalis*: comparison with Cephalochordata and Vertebrata**

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The complex organisation of vertebrate nervous system is not only the product of an ontogenetic process, but it is the result of a long evolutionary history. A disclosure of this history can help to understand i) which developmental processes are more ancient and constitute the basis of vertebrate nervous system formation; ii) which processes evolved and/or superimposed in a second time. Ascidians belong to Tunicata subphylum are a very good model for these studies, because they are Chordata and, according to recent studies, may be considered the group phylogenetically closest to vertebrates. Ascidians and vertebrates share a tripartite organisation of the central nervous system, including an anterior region, domain of *Otx* genes, a central region which express *Pax 2/5/8* and a posterior region regulated by the expression of *Hox* genes. Within this structural regionalization, during differentiation single neurons acquire an important phenotypic character given by the neurotransmitter released to excite or inhibit the target cells. We identified the serotonergic, gabaergic and dopaminergic neurons in developing central nervous system of *Ciona intestinalis* by studying the expression pattern of genes codifying the neurotransmitter synthesis enzymes. We showed that some functional regions are conserved among the CNS of ascidian larva, of the amphioxus and of vertebrates. This allowed us to hypothesize that these regions were already present in the ancestral chordate.

#### ***Citcf* and pigment organs formation during *Ciona* embryogenesis**

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The Wnt signaling cascade plays an important role during embryonic patterning and cell fate determination and is highly conserved throughout evolution. Factors of the TCF/LEF HMG domain family (Tcfs) are the downstream effectors of this signal transduction pathway. To study the function of TCF during ascidian embryogenesis, we attempted to isolate the *Ciona* counterpart(s). Our data indicate that *Ciona* genome contains a single TCF gene, *citcf* (as confirmed by the analysis of the annotated genome) compared to the mammalian genome that harbours four TCF family members. *In situ* hybridization experiments indicate that *citcf* is broadly expressed at the early stages of development; from early neurula stage *citcf* signal becomes localized to the two pigment cell precursors. Block of *citcf* function, by morpholino-antisense injection, strongly suggests its involvement in pigment cells formation; on the other end, transcriptional regulation analysis, by electroporation method, demonstrates that a 2.0 kb *citcf* promoter region, upstream from the TATA box, is able to drive the tissue specific spatial expression of reporter gene. By a comparative analysis with *Ciona savignyi* we have identified a shared element of about 0.3 kb, upstream from the *Ci/cstcf* genes, able to reproduce the pattern obtained with the 2.0 kb region. Deletion constructs are currently under investigation, in order to further restrict the 0.3 kb minimal promoter fragment and to identify the core elements, controlling *Ci*-TCF expression, and then the transcription factor(s) able to bind to this sequence. The homologous *Ciona* factors will then be searched, by blast analysis against the annotated genome, fished in the cdna collection, present in our Laboratory, and tried both *in vitro* and *in vivo* for their ability to interact with *Ci*-TCF promoter and transactivate the reporter gene

#### **Angiogenic-like mechanism in the colonial circulatory system of *Botryllus schlosseri***

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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 by means of mechanisms of tubular sprouting. We evidenced that the regeneration of experimentally ablated areas of CCS occurs by the same sprouting mechanism. In the two cases (normal growth and regeneration, the same histogenetic mechanism and homologous factors and receptors are shared. Strong similarities in organization (e.g., simple cubic epithelium) and cell structure (e.g., extension of filopodia) in the apexes of sprouting vessels during normal growth and regeneration were observed. Immunohistological responses to anti-PCNA - a marker of cell proliferation- and antibodies against vertebrate angiogenic growth factors (VEGF, EGF, FGF-2) and receptors (VEGFR-1, VEGFR-2, EGFR), reveal that cell proliferation and the same angiogenic signals take part in corresponding

sprouting regions of CCS during normal development and regeneration.

Sprouting is the most common and best-known mechanism of vertebrate angiogenesis, but it is also found in other developing structures, such as nerves, and in *Drosophila* tracheas. All our observations show that correspondences exist between the CCS sprouting modality of *B. schlosseri* and angiogenic sprouting in vertebrates, during both normal development and regeneration, and support the idea that this morphogenetic mechanism was co-opted during the evolution of various developmental processes in different taxa.

#### **Effects of Paraquat on the development and metamorphosis of *Phallusia mammillata* and *Ciona intestinalis* (Asciacea, Tunicata)**

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Paraquat (1,1'-dimethyl-4,4'-bipyridylum) is an herbicide largely employed in agriculture, the use of which has been authorized in 120 Countries. Several toxicological studies demonstrated that this substance has neurotoxic effects on numerous animal models and causes symptoms similar to those observed in patients with Parkinson's disease. We exposed *Phallusia mammillata* embryos at different concentrations of Paraquat until they reached the swimming larva stage, then we immunolabeled their nervous system with anti  $\beta$  tubulin antibody, in order to analyze the malformations eventually induced by the treatments. Treated larvae showed dose-dependent alterations of the ocellus and of the fibres that innervate the otolith. The gravity of the malformations decreased when the embryos were treated with both Paraquat and ascorbic acid, suggesting that oxidative stress is involved in the onset of the observed malformations. Moreover, we observed that treated larvae survived for more than four days, but they could not metamorphose. Exposure to Paraquat had similar effects on *Ciona intestinalis* embryos. We characterized Paraquat induced phenotype in *C. intestinalis* larvae by *in situ* hybridization with marker genes of different neural populations in order to evaluate the specificity of action of this neurotoxic agent. Treated larvae showed a drastic reduction of gabaergic neurons, while the number of dopaminergic neurons was not affected by the treatments. These results are in contrast with the supposed effects of Paraquat on humans. In fact this herbicide is suspected to be involved in the etiology of Parkinson's disease, a pathology characterized by progressive loss of dopaminergic neurons of the substantia nigra.

#### **Role of nitric oxide during *Ciona intestinalis* development**

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Nitric oxide (NO) plays an important role in fertilization and development of some marine organisms, including sea urchins, marine snail and ascidians. We have recently reported that in *Ciona intestinalis* NO is involved in larval development and metamorphosis. The spatial patterns of nitric oxide synthase expression, as well as NO detection, during larval development are very dynamic, moving rapidly along the body in very few hours, from the anterior part of the trunk to central nervous system, tail and juvenile digestive organs, thus suggesting the involvement of NO in many processes. In particular, NO regulates tail regression acting on caspase-dependent apoptosis.

To further investigate the role of NO during *C. intestinalis* development we focused our attention on NO signalling during metamorphosis. Experiments have been carried out to examine how the modulation of this signalling affects tail resorption and subsequent juvenile development. Parallel experiments have been also performed to investigate the possible involvement of NO in the embryonic development. To this aim we have examined the spatial expression patterns of nitric oxide synthase and NO detection in embryos at different stages as well as the effect of endogenous NO levels modulation on development

#### **Mathematical models for excitability of egg cells**

##### **PG Reas**

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The excitable systems play a very important role in biology and medicine. Phenomena such as transmission of impulses between neurons, the cardiac arrhythmia, the aggregation of amoebas, the appearance of organized structures in the cortex of egg cells, all derive from the activity of excitable media. In the first part of this work a general definition of excitable system is given; we then analyze some cases of excitability, distinguishing between electrical, chemical and mechanical excitability and comparing experimental observations with simulations carried out by appropriate mathematical models. Such models are almost always formulated by partial differential equations of "reaction-diffusion" type and they have the characteristic to describe propagations of electrical waves or chemical and mechanical waves (propagation of Ca waves and mechanical waves in the endoplasmic reticulum).

The aim is to put in evidence that the biological systems can show not only excitability of electrical type, but also excitability of chemical and mechanical nature, which can be observed in the first steps of development of egg cells.

#### **Session 2. Phylogenesis and microevolution**

##### **The fast evolutionary dynamics of ascidian mitochondrial genome: an exception to the general deuterostome evolutionary trend**

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The availability of more than one thousand sequences of mitochondrial genomes (mtdna) of Metazoa provides an almost unique opportunity to decode the mechanisms of evolution of an entire genome in a phylogenetic framework.

We have compared several structural features of the ascidian mtdna (gene content, genome size and architecture, and gene strand asymmetry) to that of other metazoans, focusing also on comparisons at congeneric level, as analyses at short evolutionary distances reduce the risk of saturation effects in the observed genomic changes. The current data show that ascidians exhibit a degree of mtdna variability very high and comparable to that of non-deuterostome groups. Indeed, a trend toward stabilization of the mt genomic features has occurred in most deuterostomes and has been exacerbated in vertebrates, where gene content, genome architecture and gene strand asymmetry are almost invariant. On the contrary, ascidians show a gene strand asymmetry and a variability in mtdna architecture similar to non-bilaterians and lophotrochozoans, suggesting that these features are primitive, rather than a derived traits of the mtdna. In addition, our data highlight that the high degree of rearrangements in mtdna architecture found in ascidians, as well as in enoplean nematodes, is due to translocations of both RNA and protein-coding genes, while changes in genome architecture are mostly due to the variation of number/position of trnas in taxa with moderate/low rearrangements. The variability observed in congeneric species significantly recapitulates the overall mtdna evolutionary dynamics observed at higher taxonomic ranks, especially in taxa with high genome plasticity and/or fast nucleotide substitution rate. Moreover, congeneric comparisons appear quite promising to investigate in detail mtdna evolutionary mechanisms.

##### **One ring to divide them all: mitochondrial genomics unveils two cryptic species in *Ciona intestinalis***

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The circular mitochondrial genome (mtdna) of metazoans represents a rich source of genetic markers for phylogenetic analyses at many taxonomic levels. Both sequences and genome-level features, such as gene arrangement, have been used to resolve deep-level phylogenetic relationships, whereas single mitochondrial genes or regions are commonly analysed in population genetic studies.

We used a mitogenomic approach, based on the comparison of several genome-level mitochondrial features, to unambiguously demonstrate the existence of two cryptic species in the ascidian *Ciona intestinalis*, a model chordate whose taxonomic status of a single species has been recently questioned. A comprehensive comparative analysis between the mtdna of the two putative cryptic species revealed significant differences in gene order, size and number of non-coding regions,

compositional features, and evolutionary rate of protein-coding genes. These mitochondrial features are clearly incompatible with intra-species variability, and strongly suggest the existence of the two cryptic species. Furthermore, our approach allowed to set two PCR-based diagnostic tests for the discrimination of the cryptic species without recourse to morphological analyses, demonstrating that mtDNA represents an accessible and powerful tool to be used both in routine analyses and in high-throughput screenings.

### **Phylogenetic conservation of CSF-related genes in the ascidian *Ciona intestinalis***

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In all vertebrates, mature oocytes arrest at the metaphase of the II meiotic division, while some invertebrates arrest at metaphase I, others at pronucleus stage. Fertilization induces completion of meiosis and entry into the first mitotic division. How the different mechanisms underlying meiotic regulation evolved is very far from being clarified. In the past decades, several experimental models have been considered from both vertebrates and invertebrates in order to shed light on the peculiar aspects of meiotic division, such as the regulation of the cytosolic factor (CSF) and the maturation promoting factor (MPF) in metaphase I or II. Some of these questions remain elusive and can be approached by the introduction of new experimental models. In the recent past, we proposed the oocytes of ascidian *Ciona intestinalis* as a new model to study the meiotic division both at the physiological and molecular level. Here, taking advantage of the recent publication of a draft copy of *C. intestinalis* genome, we present a phylogenetic analysis of key genes involved in the control of meiotic completion after fertilization, such as Cdc2/Cyclin B and MAPK/mos components of MPF and CSF, respectively. The presence of *c-mos* homolog in *C. intestinalis* genome suggests a regulation of metaphase arrest in ascidians different than in other invertebrates, where this gene is not conserved. We further investigated the regulation of CSF by demonstrating that both CSF and MPF inactivation, at the exit of metaphase I, are independent from protein synthesis. In fact, oocytes loaded with emetin completed meiosis I after fertilization, similarly to control oocytes, as demonstrated by MPF decrease and extrusion of the first polar body. This result indicates the absence of short-lived factors that regulate metaphase stability, as in other invertebrate species. In addition, antibody raised against the *Xenopus* homolog of Mos are able to abolish *C. intestinalis* CSF activity. Finally, MAPK enzymatic activity sharply decreases after fertilization, as confirmed by a MAPK specific antibody that was able to recognize the active, phosphorylated form of the kinase. The results obtained indicate that meiotic regulation in *C. intestinalis* resembles that of vertebrates, such as *Xenopus*, more than those of other invertebrates.

### **Advancing forward genetics in *Ciona intestinalis* sp. A.**

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Our aim is to elucidate the cellular, molecular and evolutionary basis of pattern and differentiation in animal body plans. We combine classical and modern techniques for understanding gene functions during embryonic development by centering our activity on *Ciona intestinalis* (Ascidacea). Herein, we report about the implementation of forward genetics approaches. The primary objective of forward genetics is a low-cost resource of mutants with interesting phenotypes to be rapidly mapped and identified at the molecular level, a concept which applies to a limited number of model species in which mutagenesis techniques allow to compare phenotype and genotype in great detail. We are currently analyzing several aspects that are essential for the establishment of a phenotype-driven methodology. Among them are the existence of two cryptic species, as shown by independent species concepts, the identification of a source of mutants, the construction of high-resolution genetic linkage map, the generation and culturing of inbred or semi-inbred strains, a protocol of sperm cryopreservation for the optimization of line management, and the characterization of genetic and morphological variation in natural populations. Also, we are studying the phylogeographic structure of *C. intestinalis* at global and local scale by using different molecular markers, with the aim to examine how historical, geographical and environmental factors influence the distribution of morphological and genetic polymorphism in a model chordate.

### **Shuttling and rRNA processing of pre-ribosomal subunits in the ascidian *Ciona intestinalis***

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Ribosome biogenesis in eukaryotes is one of the most challenging topics in molecular and cellular biology of the last ten years. Although different steps of pre-ribosome maturation has been elucidated in the last years, some intriguing facets remains still unresolved. Among these questions, the different fates of the two sub-units during ribosome biogenesis, and the reason why different maturation steps of these particles occur in different cellular compartments.

It is generally acknowledged for all the eukaryotes that the two different ribosomal pre-subunits (pre-40S and pre-60S) undergo to different fates during their late maturation steps. The smaller one (containing a 21S rRNA) is exported to the cytoplasm where its maturation is completed, whereas the pre-60S particle (containing a 28S rRNA) completes its maturation in the nucleus, and successively is exported in the cytoplasm as a mature particle. To the contrary of the ribosome maturation pattern up to now proposed, we found in the ascidian *Ciona intestinalis*, as well as in sea urchin and human cells, that pre-ribosomal subunits maturation is a concerted, parallel processing

and shuttling mechanism involving both the two pre-ribosomal particles; this mechanism includes a cytoplasmic rRNA processing event never described before. Primer extension experiment on total RNA extracted separately from nuclei and cytoplasm of *Ciona intestinalis* 4/8 blastomeres embryos, reveals the presence of the same “large” rRNA precursors both in the nucleus and in the cytoplasm of these cells. The comparison with what occurs in the sea urchin *P. lividus* (left panel) and in human white blood cells (not shown), allowed us to demonstrate both that the shuttling/processing model is substantially different with respect to the one proposed, and that the shuttling/processing events we demonstrated work probably in all the eukaryotic species.

### **Frank homologies between invertebrate and vertebrate chordates revealed through the neurophysiology of swimming in *Ciona* tadpoles**

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With the recent sequencing of the genome of *Ciona intestinalis* there has been an increase in interest in ascidians as models to study the evolution of chordates. One way to do this is to look at the similarities and differences between genes and families of genes at the genomic and functional level. However deeper homologies may be revealed by looking at (in addition) the function of proteins in a given physiological system which may shed light on their evolutionary pathways.

Here we report progress in our understanding of chordate locomotion and physiology through study of the neurophysiology of swimming in *C. intestinalis*.

Although *Ciona* has a sessile adult form, the tadpole-like larva swims with rapidly alternating tail beats that superficially resemble vertebrate swimming. In vertebrates, such alternating activity during swimming is generated in spinal segments and is controlled by a combination of glutamatergic excitation of motoneurons and inhibitory glycinergic interneurons which provide contralateral inhibition. Modulation of the period of swimming is achieved through GABAergic inhibition. These networks of excitatory and inhibitory neurons are known as central pattern generators (CPGs). We show that: 1) a CPG exists in the visceral ganglion and nerve chord and is excited by glutamate, 2) precise alternation of tailbeats is controlled by a glycinergic system consisting of interneurons and glycine receptors (Ci- GlyR), 3) a GABAergic system modulates swimming but does not control alternating movements. We conclude that the ‘spinal-like’ CPG and the ‘use’ of glycinergic and

GABAergic inhibition for the control and modulation of swimming were ancestral chordate innovations.

### **Session 3. Immunity**

#### **Stem cells and chimerism in colonial ascidian**

##### **A Voskoboynik**

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Natural chimerism is the coexistence of cells of two genetically distinct organisms in one individual. It is a common phenomenon, which can be detected in a wide variety of multicellular organisms, including vertebrates. In mammals, natural chimerism is usually established during pregnancy between the mother and the fetus or between fetuses in multiple embryos pregnancy. Colonial marine ascidians, like *Botryllus schlosseri*, may serve as evolutionary model system to vertebrates chimeras. In these organisms, pairs of allogeneic colonies can establish a natural chimerism upon physical contact. The ability to create a chimeric entity between these colonies is determined by a single, highly polymorphic, fusion / histocompatibility locus (Fu/HC). Colonies that share at least one allele in their Fu/HC locus (mainly kin under *in situ* conditions) would fuse upon contact. A pair that does not share any Fu/HC allele would not. Following fusion, cells transmigrate between colonies and, in some cases, replace the germline and/or the somatic tissues of the host (termed as germline and somatic cell parasitism respectfully). The replacement of host tissues by a donor genotype is pre-determined genetically and follows hierarchies of “winner strains” replacing “loser strains” tissues.

In both mammals and ascidians, natural creation of a chimera entity is restricted to kin; long-term chimerism can be established by stem cells; and tolerance or intolerance state to donor tissues can be mediated by chimerism. While several studies and observations across different species, tissues and systems link chimerism to tolerance, its actual role in tolerance induction or maintenance is yet unknown. Here i'll review the chimerism phenomenon in mammals and ascidians, discuss the possible role of stem cells as mediators of chimerism and the possible role of cells chimerism as mediators of tolerance. Future studies, which monitor the dynamics of chimeric donor cells within the host, identify the factors that support or inhibit survival and proliferation of donor cells and its affects on tolerance or intolerance should shed new light on this widespread natural phenomenon.

##### **Morula cells, phenoloxidase and DOPA-containing proteins in the compound ascidian *Botryllus schlosseri***

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Morula cells (mcs) represent the most abundant circulating haemocyte type in the compound ascidian *Botryllus schlosseri*. They are involved in defence reactions as they: i) can recognise alien substances and cells and induce cytotoxicity; ii) are the effectors of the cytotoxic rejection reaction which occurs between contacting, genetically incompatible colonies.

A main role in MC-related cytotoxicity is exerted by the enzyme phenoloxidase (PO) which converts polyphenol substrata to quinones; the latter, in turn, polymerise to form melanins.

In the present research, we carried out new spectrophotometrical and cytochemical analysis to investigate further the behaviour of PO and the nature of its substrates. Results confirm that PO is located inside MC vacuoles. In addition, immunocytochemical analysis indicate that mcs contain quinones which probably represent ready-to-use cytotoxic molecules, likely deriving from the oxidation, by PO, of DOPA-containing proteins. In addition, small DOPA-containing peptides, called tunichromes, are likely present inside mcs.

#### **VCBP genes in *Ciona intestinalis*. I. Structural analysis**

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The pivotal genes encoding for adaptive immunity molecules, such as the major histocompatibility complex (MHC) class I and II, T-cell receptors, or dimeric immunoglobulins, have not been identified in jawless vertebrates, protochordates and in non-chordate invertebrates. However, in an attempt to identify adaptive immune-like genes, a secretion-signal-peptide-selection-based approach in the amphioxus *Branchiostoma floridae*, allowed the identification of five gene families encoding secreted proteins characterized by a pair of N-terminal immunoglobulin variable domains (V) and a single C-terminal chitin-binding domain. These families are distinguished by sequence differences in their V regions. The high degree of germline polymorphism, the chimeric immunoglobulin-lectin structure of vcbps and their tissue-specific expression, are all consistent with involvement of these molecules in immune recognition. Vcbps may reflect structural characteristics of an important transition between non-rearranging innate pattern-recognition molecules and the conventional adaptive immune receptors.

In an attempt to add further evidences for the presence of alternative solutions for both innate and adaptive immunity along the phylogenetic tree, a search for VCBP genes has been carried out in *Ciona intestinalis* genome and EST libraries. This resulted in the identification of three VCBP-like genes, Ci-VCBP1, Ci-VCBP 2 and Ci-VCBP 3, encoding three proteins of 349 (Ci-VCBP1) and 338 (Ci-VCBP2 and Ci-VCBP3)

aminoacids. The analysis of the deduced aminoacid sequences indicates that Ci-VCBP2 and 3 exhibit 72 % identity, which is about 29 % when Ci-VCBP1 is compared with Ci-VCBP2 or Ci-VCBP3. The domain structure analysis of the three gene products revealed the presence of a signal peptide sequence, two immunoglobulin domains and a chitin-binding domain.

A very preliminary analysis carried out by PCR with degenerate oligonucleotides, designed on the alignment of the three nucleotide sequences, allows us to exclude the presence of other paralogous genes in *C. intestinalis* genome. Furthermore, PCR analysis performed with cdna from different individuals evidenced the presence of limited allelic polymorphism.

#### **Molecular events triggered by the interaction between the *Ciona intestinalis* anaphylatoxin and its specific receptor**

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Many molecules belonging to the arms of the innate immune system have been found scattered in invertebrates at different levels of the phylogenetic tree. In this context, a major breakthrough has been the identification in the echinoderms, in the urochordates and in the cephalochordates of gene homologs of C3, a multifunctional protein that plays a central role in the Complement System. In mammals, C3 activation produces two bioactive proteolytic fragments: C3b responsible of the opsonic pathway, and the anaphylatoxin c3a, a potent mediator of inflammatory reactions.

We have demonstrated that in *Ciona intestinalis*, like in mammals, the bioactive fragment c3a, released from C3 during complement activation, promotes hemocyte chemotaxis, thus suggesting an important role for this molecule in inflammatory processes. Cic3a exerts its functional activity through the specific binding to a cell surface G protein-coupled seven-transmembrane receptor (cic3ar), present on granular and hyaline amoebocytes, which has been recently identified and sequenced for the first time in an invertebrate species by our group.

In an attempt to better characterize the cellular and molecular events triggered by the ligand-receptor (cic3a-cic3ar) interaction, we have focused our attention on the receptor internalization process, which is an important control mechanism described for G protein coupled receptors. We have analyzed by confocal microscopy the dynamic process of internalization of the cic3ar induced by the binding of cic3a in the form of the C-terminal eighteen aminoacid peptide (cic3a<sup>59-76</sup>) of the cic3a anaphylatoxin. The kinetic of the overall process has been monitored by fixing hemocyte samples at intervals between 0 and 30 minutes, and by immunostaining, performed with an anti-cic3ar antibody and an anti-rabbit igg FITC-conjugated secondary antibody. Preliminary results indicate that cic3ar, following the cic3a<sup>59-76</sup> stimulus, is internalized in few minutes, and, in 30 minutes, recycled to the cell surface, made again available for cic3a binding.

#### **Immunomodulatory soluble factors in the compound ascidian *Botryllus schlosseri***

## **A Menin, L Ballarin**

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Cytokines are proteins with immunomodulatory activity acting in a paracrine or autocrine way. Most of them are produced and secreted by activated immunocytes after the recognition of non-self and are involved in several immunological processes such as inflammation, apoptosis, clearance of effete cells and corpses, cytotoxicity and phagocytosis.

Today the characterization of invertebrate cytokines represents one of the major topics of interest in immunobiology and, although the presence of molecules sharing homologies with Vertebrate cytokines is still a matter of great debate, the presence of endogenous immunomodulatory molecules was indirectly demonstrated in various invertebrate taxa such as molluscs, anellids, echinoderms and tunicates.

In tunicates, the presence of molecules able to influence the behaviour of immunocytes has been reported in both solitary and colonial ascidians. In the compound ascidian *Botryllus schlosseri*, the presence of soluble opsonins released by phagocytes as well as the presence of molecules able to enhance yeast phagocytosis, modulate cytotoxicity and cross-reacting with antibodies raised against mammalian pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\alpha$  has already been described.

In the present report, we investigated the presence and the behaviour, under various conditions, of molecules recognised by antibodies raised against mammalian cytokines. Results confirm the presence of soluble peptides, upon the recognition of foreign molecules, able to increase the phagocytic index of *B. Schlosseri* and recognised by antibodies anti- TNF- $\alpha$  and IL-1 $\alpha$ . The rise of phagocytosis is significantly decreased in presence of specific sugars such as galactose, rhamnose and sorbitol. The same behaviour was observed in haemoagglutination assays where the presence of glucose and rhamnose lowered the haemoagglutination titre.

This suggests that lectins, able to modulate the immune responses, are secreted by stimulated immunocytes.

## **VCBP genes in *Ciona intestinalis*. II. Gene expression analysis**

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It has been found that VCBP genes of *Branchiostoma floridae* are selectively expressed in scattered cells of the intestine, a digestive tract continuously exposed to a diverse range of potential pathogens. The overall size of these gene families together with their extensive polymorphism may be associated with specific V-directed recognition of foreign antigens. Furthermore, it has been reported that some chitin-binding proteins found in invertebrates have an antimicrobial and antifungal role. Hence, vcbps could be considered bifunctional molecules with features of the adaptive immune receptors combined with innate immune functions.

In this context, due to its phylogenetic position, the study of *Ciona intestinalis* vcbps may provide interesting indications. This, together with the possibility of the easy handling of both embryonic stages and adult tissues in *C. intestinalis* could allow a more extensive analysis of the gene expression and function. Whole mount *in situ* hybridization experiments of Ci-VCBP1, Ci-VCBP2 and Ci-VCBP3 carried out on young adults have demonstrated that the genes are exclusively expressed in the stomach. *In situ* hybridization on stomach sections confirms and extends these results providing also a more exhaustive analysis at cellular level. Ci-vcbps expression is enhanced by challenging the animals, or blood cells, with bacterial lipopolysaccharides. Preliminary whole mount *in situ* hybridization experiments carried out on larva stage indicate that Ci-vcbps expression is localized in defined regions of the endoderm.

## **Inflammatory responses of the ascidian *Ciona intestinalis***

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Innate immune system responses include acute inflammatory and granulation tissue production phases. Cell migration, phagocytosis, encapsulation, tissue injury, matrix production, endothelial and epidermis activity, coordinate hemocyte stimulation, and wound repair have been described in *Ciona intestinalis* following body wall challenge with LPS. Several reports suggest that in invertebrates immune system, cell proliferation, phagocytosis and chemotaxis are regulated by cytophilic humoral molecules with functional similarities to vertebrate pro-inflammatory cytokines (IL1, IL6, TNF) and galectins. Here we show that like in mammals this inflammatory agent promptly challenges several cellular and molecular responses including enhanced serum lectins with IL-1 epitopes, TNF, and type IX-like collagen.

Galectin-like molecules (Ca<sup>2+</sup>-independent, specific for D-galactose and  $\beta$ -galactosides) with opsonic property can be enhanced as a response to a body wall wound and their release further stimulated by LPS. The western blot pattern and immunohistochemical methods display oligomers with hrIL1 $\alpha$  epitopes expressed by hemocytes, endothelial tissue and present in hemopoietic sites. These lectins can be promptly released by hemocytes challenged *in vitro* with LPS; both cell lysate supernatants and hemocyte culture medium displayed hemagglutinating and opsonic activities inhibited by  $\beta$ -galactosides, and anti-hrIL1 antibodies. Although a Percoll density gradient separation method showed that several hemocyte types contain and release  $\beta$ -galactoside-specific molecules, assay of enriched hemocyte populations suggest that amoebocytes are the primary source of these molecules.

For the first time we show CiTNF $\alpha$  to be constitutively expressed in the ascidian inflamed body wall and hemolymph, and upregulated by *in vivo* LPS inoculation. cDNA and deduced aminoacid sequence as well as LPS challenged gene expression demonstrate unequivocally the involvement of soluble and cell bound CiTNF $\alpha$  forms in the ascidian

inflammatory response. Sequence similarities with vertebrate TNFs include this cytokine into the TNF family. A prompt (2-4 h) enhanced CiTNF gene expression in the inflamed body is shown: *in situ* hybridization assays, cytometry and immunohistochemical methods support the involvement of pharynx and circulating hemocytes in the inflammatory response. Immunoblotting assay with anti-CiTNF specific or anti-hrTNF antibodies revealed that hemocytes contains a 43 kDa CiTNF whereas a 15 kDa TNF is released into the serum hemolymph suggesting that, like in mammals and fish, CiTNF $\alpha$  cell bound homotrimer may be processed to a monomeric soluble form. Densitometry analysis confirmed that CiTNF $\alpha$  protein expression is modulated by the LPS challenge.

Enhanced transcript of a collagen with FACIT structural features (Ci-typeIX-Col), in hemocytes, epidermis and migrating cells is also shown, while flow cytometry with specific antibodies, raised against an opportunely chosen Ci-typeIX-Col synthetic peptide, displays fibroblast-property of hemocytes challenged *in vitro* with LPS (at 4 hrs). *In situ* hybridization assay and immunocytochemistry identified collagen expressing hemocytes and disclosed epidermis gene expression in the time-course of the inflammatory reaction presumably involved in the inflammatory granulation phase.

Finally, the expression of a phenoloxidase component appears to be stimulated by inflammatory stimuli. In conclusion, the inflammatory response of *C. intestinalis* is characterized by components homologous (TNF, C3-like, collagens) and analogous (galectin-like) to the vertebrate ones.

#### **Tunicate immunocytes can be cytotoxic toward foreign cells**

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Tunicate immunocytes can be cytotoxic toward foreign cells, and cytolytic molecules ("lysins") have been revealed *in vitro* by using erythrocyte targets. In *Ciona intestinalis* the hemocyte cytotoxic activity has been examined towards mammalian erythrocytes in a medium isosmotic to the hemolymph containing 10 mM Ca<sup>2+</sup> (TBS). Unilocular refractile hemocytes (URGs) release cytotoxic factors inhibited by sphingomyelin in a plaque-forming assay. To separate the lysin-releasing cells from the hemolymph and characterize lysins, a discontinuous Percoll gradient was performed and hemocyte populations were separated in 6 bands. URGs cytotoxic for RE were enriched (~40 %) in the band 5 (B5) and then lysed to obtain the supernatant. The B5-lysate supernatant (B5-HLS) showed lytic activity (~84 %) specific for RE and K562 cells whereas a lower level of cytotoxicity was found against sheep erythrocytes (SE). Such an activity was Ca<sup>2+</sup>-dependent and thermostable at 56 °C, B5-HLS also showed a Ca<sup>2+</sup>-independent hemagglutinating activity against trypsinized RE (HT 4.3) but not toward trypsinized SE. Inhibition experiments displayed that lysins and lectins could be inhibited by carbohydrates (galactose, thio-digalactoside, lactose, lactulose)

whereas sphingomyelin (2.5  $\mu$ g/ml) only inhibited the lytic activity, suggesting that lectins may be involved in membrane sphingomyelin-lysine interactions. To check for the enzymatic nature of the lysins, phospholipase A2 inhibitors such as dibucain and quinacrine were assayed. The experiments showed that both the molecules were inhibitors of B5-HLS cytotoxic activity suggesting the involvement of a Ca-dependent phospholipase A2 activity. A cytotoxic mechanism based on phospholipase A2 activation due to lectin-sugar interactions is discussed as a model. Lysins against RE and K562 cells with the same properties including sugar and phospholipase A2 inhibition, can be promptly (within 3 h) released *in vitro* by URGs in a culture medium suggesting that activated cells could participate in the defence response exerting a cytotoxic role.

#### **The prophenoloxidase system is activated during the tunic inflammatory reaction of *Ciona intestinalis***

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In invertebrates, the "prophenoloxidase activating system" involved in immune responses, is challenged by  $\beta$ -1,3 glucans or lipopolysaccharides through a limited proteolysis due to serine proteases. Phenoloxidase (PO) is usually synthesized as proenzyme (prophenoloxidase, proPO) and, upon activation, it plays a key role in humoral immune response and melanization processes. PO activity in the tunic tissue of *Ciona intestinalis* following LPS intratunic injection was examined. Tunic homogenate supernatant (THS), assayed with Dopa-MBTH reaction, displayed Ca<sup>2+</sup>-independent PO activity that was raised by LPS and further enhanced by proteases. Specific inhibitors (tropolone, phenylthiourea, diethylthiocarbamate) supported the specificity of the reaction. Assay with soybean trypsin inhibitor suggests that, in the tunic, proteases diverse from serine proteases could also be involved in the activation pathway. *In vivo* experiments were carried out by injecting isosmotic medium or LPS, and THS assayed for its PO activity. ANOVA analysis of the time course profiles showed that LPS was more effective in activating proPO. To disclose the PO response at the injured site, an assay with Dopa-MBTH was performed *in vitro*. Quinones were mainly contained in the tunic matrix enriched with inflammatory cells around the injection site. Microscopy observations and immunohistochemistry with anti-CinPO-2 antibodies showed granulocytes and unilocular refractile granulocytes containing PO, whereas rare morula cells were stained. In THS zymograms (SDS-PAGE), PO activity linked to 90 and 120 kDa bands was observed as an effect of LPS injection, whereas the density of the 170 kDa PO was weak. In addition a third presumptive PO enzyme (CinPO-3) containing the CinPO-2 peptide was identified in the recent *Ciona* genome version. The possible involvement of a presumptive CinPO-3 similar to CinPO-2, as predicted by *in silico* analysis with BLAST (PAM 30 scoring matrix) of *Ciona* genome sequences (JGI V2), is

discussed. Presumably, LPS stimulated the production and dimerization (120 kDa) of CinPO-3 (66 kDa). In conclusion, the activated proPO system includes several POs distinguishable in their size contained and presumably released by tunic inflammatory cells and hemocytes of the pharynx bars.

#### **Isolation, characterization and expression analysis of a collectin in Tunicate *Ciona intestinalis***

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Collectins are a family of calcium-dependent (C-type) lectins characterized by four functional domains: a short amino terminal 'tail' domain, a collagen-like domain that is typified by its repeating pattern of glycine-X-Y (Gly-X-Y) amino acid triplets, a distinct neck region and the carboxy-terminal C-type carbohydrate recognition domain (CRD). Six distinct classes have been identified in vertebrates: mannose binding lectins (MBL), lung surfactant proteins A and D (SP-A and SP-D), conglutinins, serum collectin-43 (CL-43) and serum collectin-46 (CL-46). MBL can recognize carbohydrates on the surface of pathogens and then activate the central complement component, via a mannose binding lectin-associated serine protease (MASP), during acute phase responses to infection.

In order to study molecules with differential activation of gene expression during the immune response, an injection of Lipopolysaccharide (LPS) into the tunic tissue at the median body region of *Ciona intestinalis* was performed. One hour following injection animal was sacrificed and RNA was extracted and used for subtractive hybridization. RNA was reverse transcribed and cloned using *E. coli*. Sequencing analysis identified a cDNA of 863 nucleotides encoding a protein containing 221 amino acids for a molecular weight of 24426 Daltons. The sequence of this protein showed structural domains typical of collectin and a high degree of similarity with the MBL of *Gallus gallus* and human MBL 2. In addition, studies of gene expression conducted through analysis of Real Time and *in situ* hybridization, have highlighted the increase of expression of this mRNA compared to control animals that an increase of cells that express the mRNA collectin near the site of LPS inoculation.

#### **Morphological characterization and acetylcholinesterase activity in *Ciona intestinalis* hemocytes**

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The ascidian hemocytes have been reported to be involved in various functions including coagulation, nutrition, defense, inflammatory-like reaction, allogeneic reaction, tunic, gonad and germ cells

formation. In addition, some of them are known to be involved in vanadium accumulation. Hemocytes have been classified as stem cells, pigment cells, hyaline amoebocytes, granular amoebocytes, unilocular refractile granulocytes (URG), compartment cells, signet-ring cells and morula cells. Cell lineage analysis showed that hemocytes may originate from a pair of A7.6 blastomeres of a 64-cell embryo, which give rise to trunk lateral cells (TLCs). Our previous researches showed an acetylcholinesterase (AChE) activity in the TLCs of *Ciona intestinalis* swimming larva. In mammals, this enzyme has a non-classical control role in stem cell differentiation, apoptosis, defense responses and haematopoiesis. Several *C. intestinalis* circulating hemocytes were characterized by means of different staining methods, and AChE activity was identified in morula and signet ring cells. In *C. intestinalis*, this enzyme could function as an inhibitor of stem cells proliferation also related to cell differentiation. We show once again that several hemocyte forms circulate in *C. intestinalis* hemolymph some of them presumably due to differentiation steps from stem cells.

#### **Session 4. Environmental stress**

##### **Tributyltin-induced effects on MAPK signaling in ascidian embryos**

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Among the class of organotin compounds, the most well known is tributyltin (TBT). Organotin have many applications, which include use in PVC, as catalyst in chemical reactions, agricultural pesticides and antifungal treatments for textile polymers. In particular TBT is used in marine antifoulant paints to prevent the growth of organisms such as barnacles on the hull of ships. Extensive use in antifouling paints led to the widespread distribution of TBT and its breakdown products in the global marine, sediment and biota. High levels of TBT in the waters were found to have impaired reproduction, by inhibiting embryogenesis and larval development in a variety of marine organisms. Symptoms of the exposure to high levels of TBT in some invertebrates includes the development of male sexual characteristics as a penis and vas deferens by females (imposex). Ascidians are a good model for the study of embryonal development. They are also sensitive bioindicators of habitat degradation. The effects of tributyltin (IV) chloride (TBT chloride) solutions on ascidian embryos of *Ciona intestinalis* at different stages of development have been described. Previously, we carried out observations with both the light and the electron microscope on *Ciona intestinalis* embryos and larvae incubated in TBT solutions. This studies showed morphological and ultrastructural modifications of the embryos and larvae after incubation in TBT chloride at different concentrations. To understand molecular effects of TBT-induced on ascidians embryogenesis we have set out to study the effects of TBT at different concentrations, testing the activity of some protein with a basic role in embryonic development. In ascidian

embryos, a fibroblast growth factor (FGF)-like signal has been proposed to be involved in induction of notochord and mesoderm formation. A main pathway is a protein kinase transduction pathway, which includes Ras, Raf, mitogen-activated protein (MAP) kinase and extracellular signal-regulated kinase/MAP kinase (ERK). The aim of this work in progress is to understand whether the TBT exposure on ascidian embryos at different stage of development cause alterations in tyrosine phosphorylation pattern and in MAPK activity. Tyrosine phosphorylation promotes cell growth, differentiation and apoptosis, due to activity of receptor tyrosine kinases and furthermore different stressors are known to stimulate tyrosine kinase activity. At first we focused our attention on tyrosine phosphorylation pattern after ascidian embryos to different stage of development TBT treatment. Phosphorylated proteins pattern is evaluated by SDS-PAGE electrophoresis and Western blotting on protein extract of ascidian embryos incubated with TBT, using anti-phosphotyrosine-antibody directed against mammalian phosphotyrosine. Preliminary results showed a different pattern on protein phosphorylation in response to the incubation with TBT in  $\mu\text{M}$  range. Since mapks play a key role in animal responses to a wide variety of environmental stresses, we have thought to test the role of MAPK pathway proteins such as MAPK p38 (Thr 180 and Tyr 182), p44/42 (Thr 202/Tyr 204) and c-Jun N-terminal kinases (JNK) after TBT treatment.

#### **Effects of cadmium on the functionality of haemocytes from the compound ascidian *Botryllus schlosseri***

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Ascidians share a variety of circulating haemocytes differing in their morphology and involvement in various biological functions. Most of them are immunocytes, able to mount defence reactions against foreign, potentially dangerous, cells or molecules.

Metallothioneins are ubiquitous, cysteines-rich proteins which exert fundamental roles in the detoxification of trace metals such as Ag, Hg, Cd, Cu and Zn, some of which are required for normal cell metabolism.

Up to now, no molecular data are available in both solitary and compound ascidians, included the species *Botryllus schlosseri*, an important model organism for a wide variety of studies.

In the attempt to study the involvement and the role of metallothioneins in *Botryllus* immunobiology, we investigated the effects of acute exposure of haemocytes to Cd on cell functionality. Preliminary results indicate a dose-dependent negative effect on the ability to adhere of haemocytes and a decrease of

the capability of phagocytes to spread on the substrate and to trigger a respiratory burst when matched with yeast cells. A cDNA library from Cd-exposed haemocytes is now under construction from which we expect to find metallothionein sequences

#### **Toxic effect of methylmercury on ascidian (*Styela plicata*) immunocyte responses**

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Pollution by heavy metals is one of the major risk in aquatic ecosystem, where high concentration cause adverse biological effects, including changes in immune function of invertebrate and vertebrate species. In marine environment, although mercury concentration in water column and sediments may be low, filter feeding invertebrates highly accumulate this metal in their tissues. In addition, biological processes mediate the mercury methylation transforming the metal in methylmercury which is the most toxic form due to the methyl group that facilitates cell penetration and interaction with proteins interfering with their synthesis and leading to lipid peroxidation. This study shows that high methylmercury concentrations are cytotoxic for *Styela plicata* haemocytes, whereas sublethal concentrations promptly affect immunocyte responses. Moreover, haemocytes exposed to the xenobiotic present a significantly enhanced phenoloxidase activity as revealed in the haemocyte lysate supernatant compared to the control. Although the cytotoxic activity of *S. plicata* haemocytes toward rabbit erythrocytes is a PO-dependent cell-target reaction due to quinone products, it was significantly decreased by suitable methylmercury concentrations in the medium. The same xenobiotic concentrations decreased the haemocyte phagocytic activity toward yeast. In both the responses cell-target contacts could be affected by methylmercury, whereas the releasing capacity appeared to be unchanged as indicated by haemocyte PO-release in the medium. Finally, changes in haemocyte shape and spreading capacity were shown. Effects on cytoskeleton could be responsible of changes in the haemocyte morphology and spreading capacity as revealed by the microplate assay we performed. On the basis of the present results, *S. plicata* could be an additional sentinel species for heavy metal environmental pollution by using immunotoxicology tests and a microplate method that reveals cell morphological changes and spreading capacity. Similar results on the cells were obtained by assaying polluted sea water from different Sicilian coastal sites.