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REPORT OF MEETING

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Organizers: **A Ramšak**

National Institute of Biology, Marine Biology Station Piran, Slovenia

Expression study of molecular markers involved in staminality and differentiation in the colonial ascidians *Botryllus schlosseri*

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Ascidians are invertebrate chordates, members of the subphylum Tunicata that represents the sister group of vertebrates. They offer the opportunity to investigate and compare the behaviour of both embryonic and adult stem cells. Morphological data suggest the presence of undifferentiated haemocytes (haemoblasts) able to proliferate and give rise to terminally differentiated cells. Relevant studies were also carried out in the neural lineage, in which neural progenitor cells regenerate the brain after extirpation. In *B. schlosseri*, during the cyclical generation change, bud primordial cells, probably deriving from a pool of long-living stem cells, are able to give rise to the neural complex. We screened the *B. schlosseri* genome and transcriptome, looking for transcripts/genes showing similarity to vertebrate molecular markers of haematopoietic and neural stem cells. Four sequences, orthologous to mammalian transcripts considered markers of haematopoietic progenitor cells, were identified in *B. schlosseri*. They are: *bsabcg2, bscd133, bsgata1/2/3* and *bsgata4/5/6*. *In situ* hybridization on haemocyte monolayers and colony sections, resulted in labelling of cells in the sub-endostylar haemolymph lacunae. This results matches previously morphological data that identified the endostyle as a stem cell niche. Quantitative real time PCR (qRT-PCR) highlighted the over-expression of the considered genes in the mid-cycle phase of the blastogenetic cycle. During this phase, there is the formation of new secondary buds emerging from the primary buds. The high expression levels of *bsabcg2, bscd133, bsgata1/2/3* and *bsgata4/5/6* genes in the mid-cycle phase reflect the presence of undifferentiated cells involved in proliferative and differentiation events required for giving rise to the new blastogenetic generation.

For the neural lineage, we identified and characterised two transcripts orthologues of vertebrate neural stem cell markers (BsSox2 and BsMsi2). We also studied the expression, during the blastogenetic cycle, of a panel of genes already known to be involved in ascidian larvae neurogenesis, i.e., orthologues of Pax2/5/8, Hox1 and Hox3. ISH with riboprobes for BsSox2, BsMsi2, BsPax2/5/8, BsHox1 and BsHox3 revealed a common labelling in the endostyle niche. The presence of *bssox2, bsmsi2, bspax2/5/8, bshox1* and *bshox3* transcripts in the cells of the region known to be a stem cell niche, led us to conclude, not only that our probes identified undifferentiated cells but even that in *B. schlosseri* are probably present a single population of pluripotent stem cells that could differentiate into haematopoietic or neural cells. The qRT-PCR, showed an high expression level in the mid-cycle phase of all the putative neural markers considered. In this phase new secondary buds are produced from primary buds. Each new bud needs its own neural complex and this requires the proliferation of undifferentiated cells to originate neural gland rudiment and cerebral ganglion. *Bssox2*, *bsmsi2*, *bspax2/5/8, bshox1* and *bshox3* increased their expression associated with these neurogenesis events and this support their involvement in neural stem cell differentiation.

On all these sequences, we also performed a phylogenetic analysis that, always, returned us the tunicate relevant position, within the protochordates cluster, of vertebrate sister group.

Studying colonial ascidians in a landlocked country

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Tunicates belong to the Chordata phylum, phylogenetically positioned between the more basal Cephalochordata and the higher Vertebrata, of which they are considered the closest relatives. These organisms include a wide range of reproductive methods, regenerative abilities, developmental strategies and life cycles. In addition, and despite a drastically different body plan during their adulthood, tunicates have a tissue complexity related to that of vertebrates. Consequently, the study of these organisms offers a unique evolutionary perspective into the emergence of clade specific traits and the function of conserved molecular mechanisms.

In particular, colonial ascidians are established models for important biological processes including allorecognition, immunobiology and angiogenesis. Furthermore, these sessile compound organisms are the only known chordates that can undergo whole-body regeneration, whereby a fully functional adult is restored from a minute portion of their vascular system. Identifying and investigating the shared regulatory mechanisms and signalling pathways required for successful regeneration in the closest relatives of the vertebrates is of interest to regenerative medicine and ageing research. However, the current paucity in breeding infrastructures and technics limits the study of ascidians to coastal regions and thus hinders their wider scientific spreading and popularity.

Our group is interested in dissecting whole-body regeneration in the viviparous Styelidae *Botrylloides leachii*. To enable such research in Switzerland, we will develop a custom recirculating husbandry setup and identify optimal rearing parameters for the long-term culture of *B. leachii*. The designed equipment will be highly flexible to adapt to the needs of additional colonial ascidian species, as well as to those of other tunicates. The establishment of a standalone system for controlled breeding will provide the necessary platform for the worldwide spreading of colonial ascidians as model organisms. Our goal is to promote research in *Botrylloides* and thus offer a unique evolutionary perspective into chordate processes and into regeneration in particular.

Mitochondria inheritance and germ line formation in bivalves

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Doubly uniparental inheritance of mitochondria, a phenomenon known discovered in several bivalve families, defies the common rule of strictly maternal mitochondrial inheritance, commonly seen in animals. Under DUI system, paternal mitochondrial DNA is retained in male offspring, leading to heteroplasmy in males. The two emerging mitochondrial lineages are usually significantly divergent and easily distinguishable. Neither the mechanics nor evolutionary context of this phenomenon are fully understood, however the mechanism of paternal mitochondria retention in male zygotes involves sorting of sperm mitochondria at early stages of larval development, in all examined cases. Thus, the process of germ line formation in bivalves is intimately linked with DUI. Therefore, both STEM cell research and DUI research will benefit from developing cellular models of bivalvian origin.

Genomic controls of skeleton development and regeneration in the brittle star *Amphiura filiformis*

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Regeneration of adult body parts can be considered a by-product of development, however to which extent development and regeneration use the same molecular mechanisms and regulatory program is still an open question. Echinoderms are well known for their extensive regenerative abilities and well-characterized embryonic development. The aim of our study is to understand at which level the regulatory program governing the production of biomineralized skeleton is conserved between the developing embryo and the regenerating adult arm of the brittle star *Amphiura filiformis*. To understand the cellular and molecular aspects of skeletogenesis in these two developmental contexts, we used candidate and differential transcriptome approaches in conjunction with histological and high-resolution spatio-temporal gene expression analyses. We also dissected the role of the FGF signalling pathway in these two processes using a well established signalling inhibitor.

We found that 23 embryonic skeletogenic genes (transcription factors, signaling receptors and downstream differentiation genes) are also expressed in mesenchymal cells in the dermal layer of the adult regenerating arm, where skeletal spicules form. This indicates a very similar molecular signature of embryonic and regenerative sclerocytes. FGF signalling perturbation using the SU5402 inhibitor interferes with skeleton formation during both embryonic development and adult regeneration of this brittle star. A comparison of 115 genes affected by SU5402 in adult arm regeneration and during embryonic development revealed a large conservation of molecular function of FGF signalling

between those two developmental contexts. Taken together these data suggest the re-usage of the developmental regulatory program for skeleton development during regeneration in *Amphiura filiformis*. However, important differences are revealed in the early developmental genes that are not expressed in adult regeneration. Future work will aim at identifying the source of sclerocytes during regeneration and the initial factor(s) responsible of reactivating the skeletogenic program during regeneration.

Pocilloporid corals as laboratory models for research on somatic stem cells in scleratinians

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Scleractinian corals are evolutionary ancient, long-lived marine animals with high regeneration abilities, which can provide perspectives to research on stem cells, aging and regeneration processes. Reef-building colonial species are formed of a multitude of polyps connected together by coenosarc tissue. This thin (few millimeters to centimeters) tissue forms a hard calcareous exoskeleton which continuous accumulation over the years provides structural framework for many other reef species, sustaining rich marine biodiversity. Complex microbial communities are hosted within the animal tissue and skeleton, contributing an additional layer of complexity, with a major trophic role for example for the photosynthetic dinoflagellate symbionts. Despite their slow growth rates, the process of continuous coral extension via clonal budding of new polyps provides opportunities to study the involvement of somatic stem cells in tissue morphogenesis, and their commitment to various differentiated coral cell lineages. However, to this day fundamental processes in coral cell biology remain relatively underexplored. Laboratory models are needed to identify coral stem cells and investigate the balance controls between coral cell proliferation and differentiation.

Pocilloporids are widespread Indo-Pacific species with a branched growth form, and the *Pocillopora* genus contains key pioneer species for the colonization of new reef substrates, prior to the successive installation of other corals. The *Pocillopora damicornis* species complex provides laboratory models which are easy-to-grow in aquarium microcosms, and for which there exist a large pool of publically available datasets on ecology, physiology (symbiosis, biomineralization), reproductive biology, immune system and disease susceptibility. These models are frequently selected for establishing primary cell cultures, which are used for applications in short-term (few days to few weeks) *in vitro* physiology and ecotoxicology studies. Regarding 'omics' resources for *Pocillopora damicornis*, although a reference genome has not yet been released, the PocilloporaBase transcriptome database can be searched for gene transcripts, and very recent metabolomics datasets exist. We selected *Pocillopora damicornis* type *beta*, *sensu* Schmidt-Roach et *al*. 2014 (= *Pocillopora acuta* Lamarck 1916) as a model species, propagated in long-term aquarium cultures (at Aquarium Tropical du Palais de la Porte Dorée in Paris and Oceanopolis Brest), and genotyped its dominant *Symbiodinium* clade C1 dinoflagellate endosymbionts and *Ostreobium* (Ulvophyceae) microborers.

Pocillopora acuta produces brooded planula larvae, emitted periodically few days before full moon (variable number of released larvae across colonies of the same size). We used aquarium-grown, biofilm-covered larval settlement substrates, for which settlement success rates are over 75%, allowing us to study biomineralization processes and cellular turnover during larval metamorphosis and in the forming primary polyp.

Regular budding of polyps in the rapidly spreading juvenile colonies (few days to 1 month post-settlement) offer a window into morphogenetic processes, from the accumulation of cells into a locally raised tissue area to the opening of a slit around which tentacle start budding, forming the new oral disk.

This evolutionary ancient and ecologically important metazoan model should benefit from comparative approaches with more established marine or freshwater cnidarians models with advanced cellular and molecular tools, to provide new insights into the evolution of stem cell systems.

Mechanisms of cell recruitment in echinoderm regeneration: pluripotent *versus* dedifferentiated cells

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Regenerative abilities are remarkably widespread among echinoderms. Indeed, at all life stages these marine deuterostomes are capable of regenerating both external and internal body parts following self-induced or traumatic mutilations. Although two different mechanisms are usually employed to describe the regenerative processes, namely epimorphosis and morphallaxis, in the case of echinoderm regeneration the origin and fate of the involved cells are still unclear. An up-to-date overview of the cell recruitment during this process in all the five echinoderm classes is here provided in order to clarify the state of the art on this topic and therefore highlight the necessary future steps to cover this gap of knowledge.

Among stellate echinoderms, crinoids are the only group clearly displaying the recruitment of morphologically undifferentiated cells stocked in the stump tissues (*i.e.* coelom and brachial nerve) and their active migration to form a true blastema where they massively proliferate and differentiate to regenerate the lost tissues. Dedifferentiation occurs only in specific cases, such as basal arm amputations or stress situations.

Both brittle stars and starfish do not show a true regenerative blastema, apparently mainly relying on dedifferentiation phenomena with subsequent cell re/trans-differentiation. In starfish, dedifferentiation is massively employed in muscle tissues. Additionally, scarcely differentiated cells are apparently recruited via epithelial-mesenchymal transition (EMT) from distant sources (*i.e.* coelomic epithelium, pyloric caeca). The same occurs for brittle star cell recruitment with an important contribution of the coelomic epithelium as source of progenitor-like cells after EMT.

Sea cucumbers are studied mainly for nervous system and gut regeneration. In the former, the absence of "stemness" marker in the transcriptome suggests that radial nerve cord regeneration depends on dedifferentiation of the supporting cells that re-differentiate in both the same cytotype and new neurons. Massive myocyte dedifferentiation is employed during gut regeneration.

In sea urchins, damaged test and broken spines are reformed through dedifferentiation of stump cells with only minor local cell proliferation, whereas totally removed spines are regenerated via undifferentiated (pluripotent) cells.

Overall, echinoderm regeneration mainly relies on dedifferentiation phenomena rather than recruitment of pluripotent cells already stocked in the stump tissues but the precise origin and fate of the involved cells are still largely unknown. Echinoderm tissues, especially coelomic epithelium and muscles, show a high level of plasticity and cell proliferation, migration, and EMT play key roles in this process. Cell tracking, and coupled molecular and microscopy approaches will be strongly necessary to define the main challenge of echinoderm (and, in general, animal) regeneration, namely the understanding of the origin and fate of the recruited cells.

Hydra, a model for studying the impact of autophagy on stem cell behaviour

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BACKGROUND: *Hydra vulgaris (Hv)* exhibit a negligible senescence as their epithelial stem cells (ESCs) and interstitial stem cells (ISCs) continuously divide. By contrast *H. oligactis (Ho)* that undergo gametogenesis upon transfer to cold, develop an aging phenotype and die within four months (Brien, 1953; Yoshida *et al.,* 2006). To investigate the mechanisms of aging and resistance to aging in *Hydra*, we characterized two *Ho* strains, one cold-resistant (*Ho_CR*) where animals adapt and remain healthy, another cold-sensitive *(Ho_CS)* where animals age (Tomczyk *et al.,* 2015). Thus the mechanisms that lead to resistance to aging can be dissected in *Hydra.*

RESULTS: We first show that gametogenesis, which leads to a dramatic depletion in interstitial somatic derivatives, is actually not necessary for aging in *Ho_CS* animals. Indeed when we treat *Ho_CS* animals maintained at room temperature with hydroxyurea, as such as they lose their ISCs in the absence of gametogenesis, these animals also rapidly develop an aging phenotype. We previously showed that in *H. vulgaris* ESCs adapt to ISC loss by up-regulating a large subset of genes, including genes involved in neurogenesis and neurotransmission (Wenger *et al*., 2016). Therefore we reasoned that aging-sensitive animals (*Ho_CS)* might not tolerate ISC loss as a consequence of a lack of epithelial adaptation. In fact, we first found that in *Ho_CS* but not in *Ho_CR*, ESCs irreversibly lose their self-renewal potential within the first six weeks of aging. We have also investigated epithelial autophagy and found it strongly deficient in *Ho_CS*, with limited autophagosome formation during starvation, a poor inducibility of the autophagy flux measured *in vivo* upon MG132 or Rapamycin treatment, and an accumulation of p62/SQSTM1 levels. Also kinase assays show a constitutively-repressed Ulk1 activity in *Ho_CS*. Chronic Rapamycin exposure delays aging by sustaining epithelial self-renewal, but surprisingly this treatment stimulates epithelial phagocytosis without rescuing autophagy. Finally, we were able to induce aging in aging-resistant *Hv* animals by inhibiting autophagy through the silencing of an early component of the autophagy machinery *WIPI2*.

CONCLUSIONS: This study highlights the essential role of autophagy on epithelial stem cell renewal to maintain low senescence in *Hydra.* This longevity mechanism, novel in early-branched eumetazoans, values the *Hydra* model for aging studies.

Platynereis dumerlilii, a new model to study the involvement of stem cells during growth and regeneration

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Stem cells are the subject of intense research in biology and medicine. In many species, various categories of stem cells are present at post-embryonic stages and participate to processes such as growth and regeneration. The main experimental model of our team is the marine annelid worm *Platynereis dumerilii*, an emerging developmental biology model that has proven to be very useful for large-scale evolutionary developmental comparisons. *Platynereis* worms continuously grow during most of their life (a process called posterior elongation) and possess important regeneration abilities, two features that are widespread in animals, but not found in classical developmental biology models. We evidenced the presence of two populations of putative pluripotent/multipotent stem cells located in a posteriorly-localized growth zone (GZ) responsible for the continuous elongation of the body. These cells express a molecular signature

composed of about 20 genes, such as piwi, vasa and nanos, whose orthologs are known to be expressed in pluripotent somatic stem cells and primordial germ cells in other animals. *Platynereis* has also extensive posterior regeneration abilities: after amputation of their posterior part, including the pygidium (terminal part of the worm), the GZ and many segments, the worms will efficiently regenerate, from a regeneration blastema (a mass of undifferentiated progenitor/stem cells that forms at the amputation site), the pygidium and the GZ, which will in turn allow the elongation of the body. Preliminary data indicate that cells of the blastema share at least a part of the molecular signature of the stem cells of the posterior GZ. Using gene candidate and transcriptomic approaches, we currently expand with additional genes the molecular signature of the GZ stem cells and blastemal cells. Our data provide new insights about the evolution of stem cells and their involvement in growth and regeneration in animals.

Germinal stem cells in their niche in the oyster *Magallena gigas* (*Crassostea gigas*)

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At the origin of the germline, there are the germinal stem cells (GSC) and the study of the GSC is essential in animal organism in order to understand the sexual reproduction. Our work is interested in the GSC study in a mollusk, the Pacific oyster *Magallana gigas* (ex-*Crassostrea gigas*). Our original model presents some essential benefits for our researches as the genome availability and the fact that the gonad constitutes 80% of the animal weight during the sexual maturity period.

To identify and locate the germinal stem cells in the oyster, we used some conserved and classical criteria of the GSC. Firstly, we used the location criteria because GSC are found at the edge of the gonad structures, equivalent to gonadal tubules in oyster. Secondly, we combined morphological and molecular criteria with the observation of the chromatin decondensation and the labeling of a specific germ cell protein named Vasa. The chromatin appears decondensed in GSC nucleus, that denotes a low mitotic activity specific in quiescent cells as GSC (*Chiarini-Garcia and Russell,* 2001, 2002). Concerning the molecular criteria, the protein Vasa is known as a specific marker of the germinal cells including the GSC in several organisms from invertebrates to vertebrates (Juliano *et al.*, 2010). Vasa is present in oyster in germ lineage and we used a specific antibody developed against the oyster vasa protein Oyvlg (Fabioux, 2004, 2009).

These cellular and molecular criteria are not sufficient to identify for sure the putative GSC in oyster. That's why we developed an additional approach of quantitative screening on tissue sections (quantitative histology). This approach allowed us to visualize a subpopulation of putative GSC and gave us information about the nucleus shape of these cells. Looking at the ultrastructure of these cells, we identify two types of putative GSC by transmission election microscopy (with two nucleus shapes) or more probably two differentiation stages of GSC. We also became interested in the description of the specific microenvironment, named germinal niche, of GSC in the gonad. This last part will be helpful for a better identification of cells neighbouring GSC and that could be implied in the regulation of stemness and differentiation of GSC pool. Finally, to study the functioning of the germinal niche, we develop *in vitro* tools of enrichment of populations in GSC or associated somatic cells. These enrichments were performed by differential adhesion of the cell fractions of by cell sorting based on aldehyde dehydrogenase activity.

Action of beta-catenin and myc signaling in the *Hydra* interstitial stem cell system

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Myc factors are known for their roles in stem cell maintenance, cell cycle regulation, and cancer. They are in fact evolutionarily old: they evolved at the transition from pre-metazoan colonies to true metazoans and have since not dramatically changed their protein structure. Cnidarians are the only metazoan phylum together with vertebrates to show ancestral diversification of the *myc* gene family. Cnidarian genomes commonly encode at least four *myc* paralogs. In *Hydra*, two of the four Myc factors are structurally and functionally similar to mammalian c-Myc. Two *Hydra* Myc factors, however, exhibit a unique and derived N-terminal protein structure that has no homology hit in structural data bases. We have meanwhile studied structure and function of three *Hydra* Myc factors. Analysis of gene expression patterns demonstrates that the three corresponding *myc* genes are activated in interstitial stem cells and their proliferating derivatives. Two of these three genes seem to be directly regulated by Wnt-beta-Catenin signaling. Unexpectedly, *myc1* shows pronounced down-regulation in beta-Cat-transgenic and Alsterpaullone-treated polyps. This is different to mammals, where beta-Catenin is one of the best-known upstream regulators strongly activating *c-myc*. Myc1 repression by *Hydra* beta-Catenin also occurs in reporter assay experiments in vertebrate cell culture. We have started to approach functional interference with beta-Catenin and Myc1 by using transgenic approaches, siRNA, and small molecule inhibitors, and we find effects on decision making in the interstitial stem cell system. A model how beta-Catenin and Myc factors affect stem cell dynamics, self renewal and differentiation is discussed.

Metagenomics of the northeastern Mediterranean; cave and marine habitats

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Next Generation Sequencing (NGS) technology provides great amount of information about marine biodiversity as well as functional diversity and active metabolism. Metagenomic analyses using NGS technology have revealed numerous previously unrecognized microorganisms. In the present study, bacterial community of four different depths samples (0, 25, 150, 200 m) from a North-eastern Mediterranean offshore station (200 m) and a surface water sample from a seaside cave lake were studied using metagenomics amplicon sequencing technique. Targeted marine samples were micro-plastic particle-attached microorganisms and cave sample was both free-living and particle attached organisms. About 30,000 Operational Taxonomic Units (OTUs) from 695,000 sequences were observed for all the depths of marine samples and 13,000 OTUs from 162,000 sequences for the cave sample. Pseudomonadales (Proteobacteria) was the most dominant order in the particle attached marine environment, which the members of taxa are capable of degrading Polycaprolactone (PCL). Another common bacterium for marine environment was Phenylobacterium that degrades the Chloridazon herbicide. On the other hand Nitrosporia, which found as the most abundant group for the cave lake, plays a role in the nitrogen cycle by performing nitrite oxidation in the second step of nitrification. This is a first study using a culture-independent approach for identify the North-eastern Mediterranean free-living and particle-attached marine and cave habitats microorganisms.

How to identify and isolate bivalve cells for *in vitro* applications: tools, first results and perspectives

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Our laboratory has a quite long history in investigating the neuroendocrine control of various processes in molluscan species. We have developed specific bioassays in *Mytilus edulis* and *Crassostrea gigas* allowing to evaluate physiological processes of isolated cells maintained in primoculture for several days (DNA and protein synthesis, glycogen metabolism…). The presentation aims to propose a situational analysis of our background regarding the bivalve cell culture assays. One of the major difficulties concerns frequent contaminations (bacteria, fungi and protozoa). The use of antibiotics and antifungal substances in the suspensions of dissociated cells may be combined with density gradient allowing to separate cells from contaminants. Dissociation processes consist in a combination of mechanical and enzymatic treatments. Cell enrichment in one cell type relies on the possibility to identify some of these cell fractions using specific markers (antibodies, transcriptomic probes, enzymatic activities…). Three examples of cell fractionation are illustrated: 1- enrichment of oyster germinal cells of the male lineage using staput BSA gradient associated to cell identification based on ultrastructural characteristics of collected cells combined with flow cytometry (DNA quantity and mitochondrial labelling). 2- Germinal stem cells enrichment based on differential adhesion in flask assessed by expression of stemness markers (KLF4, Sox) 3- Germinal stem cells enrichment based on FACS cells sorting (ALDH activity). Proliferation and viability of cell are currently checked using trypan blue, MTT or BrdU incorporation tests. The impedance measurement is also an interesting tool for adherent cells (X-CELLigence system, Ozyme), allowing a real time, label free, sensitive cellular analysis of cell number, adhesion, viability and morphology. First attempts on *C.gigas* hemocytes provided low cell index regarding to vertebrate tumoral cells, potentially due to reduced cell size, low cell adhesion and restricted proliferative activity.

Cellular dynamics during *Clytia* medusa regeneration

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Marine metazoans show remarkable regenerative capabilities. Cnidarians, in particular, have long been shown to possess very efficient repair mechanisms. Research has so far mainly focused on the regeneration of the polyp form, with studies on *Hydra*, *Nematostella* and *Hydractinia*. In contrast, medusae were thought to have lower regenerative capacities, due to their anatomic complexity: striated muscle, a well-organized neurosensory system, and well defined organs (manubrium – feeding organ, tentacle bulbs and gonads), connected by a system of radial and circular canals. The hydrozoan *Clytia hemisphaerica* has recently emerged as a model in evolution, cell and developmental biology. Its complex life cycle, including a planula larva, colonial polyps and pelagic medusae, is controlled in the lab, and tools for functional analyses, as well as assembled genome and transcriptomes, are now available. We could demonstrate that *Clytia* medusae also possess remarkable regenerative capacities, being able to restore both their organs and body form. Mouth, gonads and tentacle bulbs harbor pools of multipotent stem cells, and show high levels of cell proliferation. Dissection and grafting experiments – each organ can survive as an autonomous unit – are allowing us to address the interplay between the stem cell pools. I will present our current advances

in the characterization of the cellular and molecular processes during *Clytia* medusa regeneration, offering a fresh perspective on our understanding of cnidarian regeneration and medusa patterning.

$GABA_B$ signaling regulates metamorphosis, neurogenesis and regeneration in the sea anemone *Nematostella vectensis*

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GABA has multiple functions in mammalians during early development and in the adult, promoting neurogenesis progenitor proliferation as well as synaptically inhibiting neurons. We found that in the basal sea anemone *Nematostella vectensis*, GABA play roles in planula-to-polyp transformation and during regeneration, which are mediated by $GABA_B$ receptor. The effects of chronic application of $GABA_B$ agonist on the developing nervous system as well on the physiology of developing planulae will be discussed.

Arachidonic acid metabolism in marine invertebrates

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Research goals: Our research group focuses on discovering novel lipid mediators (eicosanoids and other oxylipins) in marine organisms, identification of their biosynthetic routes, and characterization of genes and proteins involved in the lipid metabolism. Oxylipins, oxygenated lipid mediators formed from various polyunsaturated fatty acids, are well-established stress mediators, synthesized mainly by lipoxygenase (LOX) and cyclooxygenase (COX) in vertebrates, and by LOX-dependent pathways in plants. In soft corals, along with COX and LOX enzymes, the initial oxidation of arachidonic acid (AA) is also catalyzed by a catalase-related allene oxide synthase-lipoxygenase (AOS-LOX) and hydroperoxide lyase (HPL-LOX) fusion proteins.

Techniques used: We are specialists in biochemistry and molecular biology of fatty acid dioxygenases. In our experiments we routinely use bioinformatics for evolutionary and structural studies, clone and express enzymes in the bacterial, yeast and insect expression systems, conduct site-directed mutagenesis, purification and kinetic characterization of recombinant proteins, define their structures (X-ray crystal studies) and analyze enzymatic reactions; separate and analyze the formed products by HPLC, mass-spectrometry (LC-MS and GC-MS) and UV-Visible spectroscopy and identify novel compounds by NMR. In addition, we perform gene expression profiling by RT-qPCR. We also study biomolecular interactions by surface plasmon resonance, fluorimetry and calorimetry. Maintaining of the marine aquarium and providing a suitable environment for cultivating and propagating corals is also essential, as part of our work includes experiments *in vivo.*

Main findings: We have unraveled eicosanoid biosynthetic routes in corals (Varvas *et al*., 1999), amphipods and red algae. Specifically, we have discovered and characterized (i) 15*R*-specific COX from Caribbean coral *Plexaura homomalla* (Valmsen *et al*., 2001) and the first algal COX from *Gracilaria vermiculophylla* (Varvas *et al*., 2013) (ii) lipoxygenase (LOX) with a unique arachidonate 11*R*-specificity from Arctic coral *Gersemia fruticosa*, (iii) catalase-related allene oxide synthase- and hydroperoxide lyase-lipoxygenase (AOS-LOX and HPL-LOX) fusion protein pathways in soft corals (Koljak *et al*., 1997; Teder *et al*., 2015). Furthermore, we have defined the X-ray structure of 11*R*-LOX (Eek *et al*., 2012), analyzed the oxylipin metabolism in corals *in vivo* and shown the involvement of oxylipins in the stress response of soft coral *Capnella imbricata* (Lõhelaid et al., 2014, 2015), and determined oxylipin profiles of multiple stony corals, *Acropora millepora, A. cervicornis* and *Galaxea fascicularis* (Lõhelaid and Samel, 2018).

Our results suggest that although the profiles of lipid mediators vary among species, the enzymes involved in oxylipin synthesis are highly conserved from invertebrates to vertebrates providing an excellent tool to study complicated mammalian signaling pathways in invertebrates as model organisms.

The tunicate *Botryllus schlosseri*: a model for developmental and evolutionary studies

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Ascidians are marine, filter-feeding chordates, belonging to the subphylum Tunicata, which are considered the sister-group of vertebrates. In the last years, several solitary ascidians (such as *Ciona intestinalis* and *Halocynthia roretzi*) have emerged as model organisms to study the molecular control of embryogenesis and cell lineage. Their genome has been partially or fully sequenced and different molecular tools are now available. Colonial ascidians are less well known at molecular level. However, they offer the opportunity to study the development of clonal individuals during asexual reproduction and to compare, in the same organism and at various levels (molecular, biochemical, morphological), sexual and asexual reproduction.

Botryllus schlosseri is a colonial ascidian whose genome has been sequenced and the anatomical and developmental ontology is available. Fertilisation is internal and embryos develop inside the parent attached to placental cups. At 18 °C, larvae develops in a week and then hatch. They swim for few hours searching a substrate on which to metamorphose, in order to found a new colony. Colonies are formed of numerous, genetically identical individuals (blastozooids) undergoing cyclical generation changes: weekly, in laboratory conditions, adult zooids die and are replaced by their maturing buds (primary buds). Buds grow on the lateral walls of their parent and produce

secondary buds (or budlets). At generation change, budlets are ready to become primary buds and to produce a new generation of budlets. Budding occurs continuously within a colony, in an orderly and synchronized fashion, so that three blastogenic generation coexist: the adult zooids, the primary buds, and the secondary buds. Since embryogenesis and blastogenesis lead to formation of morphologically similar individuals, this species is interesting from a developmental and evolutionary point of view to verify whether steps in embryogenesis are repeated during blastogenesis, or if the latter is a completely new type of development.

Multipotency of adult neural stem cells in the rodent brain

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Neural stem cells (NSCs) persist in the hippocampus of most mammals and are able to generate neurons through adulthood, a process known as adult neurogenesis. Adult neurogenesis is important for spatial memory and learning, pattern separation and responses to stress and anxiety. rNSCs are more multipotent than previously thought and generate more copies of themselves and astrocytes. In pathophysiological conditions such as epilepsy they can generate reactive astrocytes that participate in the neuroinflammatory response. NSCs can also generate oligodendrocytes after genetic manipulation. The type of cell division (symmetric versus asymmetric) and the differentiation path is tightly regulated by neuronal activity with gamma aminobutyric acid (GABA) being a main mediator. In normal conditions neurogenic asymmetric cell division is predominant with a low percentage of symmetric cell division. In conditions of neuronal hyperactivation such as epileptic seizures NSCs become reactive (hypertrophic and with massive mitotic activation) and switch to symmetric cell division generating more copies of RNSCs that will differentiate into reactive astrocytes, thus abandoning their neurogenic potential and participating in the neuroinflammatory response. We are currently exploring the molecular pathways linking neuronal hyper excitation and the induction of RNSc.

Small undifferentiated cells from starfish *Asteria rubens* L.: candidates to the role of progenitor cells

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The study aims to characterize cells and protein factors involved in regeneration of tissues and organs of starfish *Asterias rubens*. Starfishes regenerate at a much slower rate than other echinoderms. We focussed on the study of coelomocytes origin, an immune/haematic system of adult *A. rubens* which is able to rapid renewal. Two types of experimental traumatic treatment, puncture wound and vast blood loss (maximal coelomic fluid draining off followed by washing the coelomic cavity with sea water) were used to stimulate coelomocyte production. The small morphologically undifferentiated cells with high nuclear-cytoplasmic ratio were proposed to be the progenitors of coelomocytes. They are comprised up to 50% of the subpopulation of cells weakly attached on the surface of the coelomic epithelium (CE) and were named CE-W. Their characteristic is the proliferative activity *in vivo* and *in vitro*.

The presence of morphologically similar cells was found in other tissues of starfish. Two types of small cells with high nuclear-cytoplasmic ratio, with densely stained nuclei and discretely stained nuclei were found in coelomic cavity, CE, axial organ, Tiedemann bodies, pyloric caeca and near-anal caeca. Stomach and rectal glands possess their own type of cells with high nuclear-cytoplasmic ratio.

Mitotic activity at extremely low level was typical of two types of cells: small cells with high nuclear-cytoplasmic ratio and larger ones possessing the visible cytoplasm. Stomach and rectal glands have their own type of mitotic cells. These data suggest that small cells with high nuclear-cytoplasmic ratio can perform their functions in different organs of starfish.

The irregular distribution of cells on the surface of the CE was demonstrated. Ultrastructural analysis of the CE revealed the localisation of small cells under the layer of ciliated cells and in the connective tissue and showed cells migration from CE into the coelomic cavity.

The proteomic study of cell-free coelomic fluid was performed by mass-spectrometry.

Ninety-one proteins were identified. Proteins were classified into 12 categories according to putative molecular function. The largest fraction of proteins was classified under “pattern recognition receptor activity”, including “carbohydrate binding”. Two other highly represented categories were “signal transducer activity” and “peptidase inhibitor activity”. Domain organisation of proteins was presented. The comparison of two types of damage was done. Proteins, specific for each type of injury, were identified. Quantitative evaluation of injury stimulated changes was performed and down-regulated and up-regulated proteins were defined. The data give us an understanding of molecular processes which take place in the CF of *A. rubens* in response to injury.

To obtain molecular markers for coelomocytes, CE and CE-W, enriched with undifferentiated cells, a comparative proteomic study is in progress. Proteins common and unique for each cell population were identified (preliminary data). Percoll density gradient centrifugation was performed to enrich heterogeneous cell

populations with specific cell types before performing proteomic analysis. We obtained 70% enrichment with undifferentiated small cells, significant separation of a mass of ciliated cells and the functional fractionation of coelomocytes. This study was supported by the Russian Foundation for Basic Research № 15-04-07798. The experimental work was performed on the base of White Sea Biological Station of the Zoological Institute, RAS, Cape Kartesh.

Molecular and cellular aspects of neurogenesis in the anthozoan *Nematostella vectensis*

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In many bilaterians, the generation of nerve cells involves symmetric and asymmetric divisions that eventually lead to a set of functionally and morphologically diverse neurons. We are studying neurogenesis in the anthozoan *Nematostella vectensis*, a member of the Cnidaria, the sister group of bilaterians. While their phylogenetic position can be informative for reconstructing early steps in the evolution of neurogenesis, their broad neurogenic potential and high regenerative capacity makes cnidarians also interesting models for understanding general aspects of nervous system development. Using transgenic reporter lines, gene knockdown and genome editing, we are trying to analyze the molecular and cellular basis of neurogenesis in *Nematostell*a. We have identified a population of *soxB(2)*-expressing neural progenitor cells which can give rise to all major neural cell classes and a population of unipotent progenitor cells that generates a particular type of putative sensory cells. By comparing the gene expression profiles of embryos with increased and decreased neurogenesis, respectively, we are now moving beyond candidate gene approaches to obtain a detailed picture of neural development in *Nematostella*.

Stem cells in marine biotechnology

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Marine Biotechnology is becoming progressively more central to delivering benefits from the sea to human health and wellbeing. The seas and oceans represent indeed a unique environment with the potential to contribute enormously to the sustainable supply of food, energy and biomaterials. It is estimated that 25% of the total number of species on Earth correspond to marine species (Mora *et al*., 2011). These have evolved mechanisms to thrive in an extremely different and hostile environment compared with land, leading to an high biodiversity reflected by the myriad of secondary metabolites (or natural products) that marine species produce to defend themselves against predators, to locate mates and to compete for resources. Many of these compounds have no terrestrial counterparts and are unique in terms of chemical structure and biological activity. Marine organisms thus provide an enormous potential for exploration of bioactive molecules for biotechnological applications. In fact, notwithstanding the high number of compounds isolated so far from marine organisms (now exceeds 28,000), hundreds of new compounds are discovered every year (Blunt *et al*., 2015). Major source of bioactive natural products are marine invertebrates among which sponge, cnidarian and mollusks are the most productive. Despite the high number of natural products isolated so far, those that have either been marketed or are under development are relatively few. Bottlenecks to overcome to reach the market are still present, as for example difficulties in harvesting organisms, low amount of natural product in producing organisms, problems in obtaining a sustainable supply of the compound, difficulties in isolation and purification procedures, ecological impact on natural populations, and insufficient investment by pharmaceutical companies (Torjesen, 2015). Notwithstanding these difficulties there has been a 'renaissance' in marine drug discovery in the last decade due to technological developments and the use of marine microbial genomics to provide biosynthetic pathways for the production of marine natural products (Glaser and Mayer, 2009). Further improvement in the biodiscovery pipeline may come from study and utilization of stem cells for biotechnological applications, e.g. for bioactive metabolite production, which may solve one of the major bottleneck represented by sustainable supply of sufficient amount of bioactive compounds to support all phases of clinical trials for new drug delivery to the market.

Tissue engineering inspired in marine organisms: current biomaterials and future persprctive on stem cell role

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Marine organisms are growingly recognized as an attractive source of inspiration for scientists and engineers. Diverse chemical compounds are being produced, from polysaccharides to biologically active metabolites, with a few examples being in the market as components of anti-tumour and antimicrobial drugs. Additionally, the morphological features of marine glass sponges influencing architectural trends and materials processing endeavours, as well as mussel foot proteins with fundamental role on the development of new wet adhesives are striking examples of marine biomimetics.

Within an extensive work being developed in our research group on tissue engineering and

regenerative medicine (TERM), combining human stem/progenitor cells with biodegradable 3D polymeric matrices towards the establishment of *in-vitro* tissue constructs, several models of marine inspiration are being explored in our lab. Most efforts are on the development of biomaterials for TERM, as well as other advanced therapies to address diseases as cancer and diabetes, following both the route of marine origin materials and the marine biomimetic approach. In this regard, examples of different marine origin polymers will be discussed, exploring different processing technologies towards the proposal of scaffolds and membranes for cell culture under TERM approaches, namely addressing: (i) marine origin collagens, isolated from skins of different fish species or squids, marine sponges and jellyfish, on the production of hydrogels and composite scaffolds; (ii) squid chitosan, with higher deacetylation degree, on membranes or porous scaffolds; (iii) seaweed sulfated polysaccharide fucoidan, not only as potential drug to tackle breast cancer but mainly as structural component of hydrogels for cell encapsulation (iii). Moreover, marine biomimetic concepts will be also explored, namely by using the marine sponges porous skeletons as Nature made scaffolds or as inspiration for the development of hierarchical structures by combining 3D printing of a support material with microfibers made of a cytocompatible biopolymer aiming to influence cell fate, as cell differentiation into a specific lineage. In this regard, the comparison of the behaviour of human and marine invertebrate stem cells may give important cues to trigger the new tissue formation and ultimately the design of advanced regeneration therapies.

The amphioxus regeneration model

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The cephalochordate amphioxus is emerging as a promising invertebrate chordate model for studies of the evolution of development ("evo-devo") and genome organisation, particularly at the invertebrate-vertebrate transition. It is also beginning to provide insight into the evolution of regeneration mechanisms: unlike most vertebrates, amphioxus shows considerable regenerative ability of all major structures (notochord, neural tube and axial musculature) even as an adult. In an effort to begin to elucidate the molecular and cellular processes underlying tail regeneration in amphioxus, we have generated transcriptomic and proteomic resources. Unexpectedly, this has uncovered a number of candidates, notably gene duplications, with interesting evolutionary histories. Here, I will present some of these new data and discuss their possible implications for the evolution of embryonic and regenerative processes. I also highlight current and future avenues of research that will improve the amphioxus model system.

***Ciona robusta* as model system for regeneration and *in vitro* cell culture**

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Ascidians, as *Ciona robusta,* belong to the phylum Chordata, subphylum Tunicata and are characterized by a larval stage and an adult stage as result of a dramatic metamorphosis. Their larval stage thus retains pluripotent cells as a reservoir for extensive changes that occur in these animals during metamorphosis. In this regard, a study has been conducted in order to address how much and which part of the larval CNS contributes to form the adult CNS during metamorphosis, by tracing cells in *Ciona* larval central nervous system (CNS). The data demonstrated that most parts of the ascidian larval CNS are maintained during metamorphosis and recruited, as stem-like cells, to form the adult CNS (Horie *et al*., 2011). In particular, this study highlighted an important role of ependymal cells as reservoir of neural stem-like cells in order to reconstruct the adult nervous network during chordate metamorphosis. *Ciona* has also retained the capacity to regenerate, although more limited compared to colonial ascidians, and this process potentially involves mechanisms of cell de-differentiation. Ciona adults, indeed, can rapidly and robustly regenerate their oral siphons (OS) as well as their central nervous systems (Jeffery, 2015a, 2015b). The stem cells involved in OS replacement are located in lymph nodes lining the transverse vessels of the branchial sac; they initiate proliferation in response to distal injuries and invade the wounded areas to form the blastemal. Recently, microarray and RNA sequencing approaches have permitted to characterize mRNA and miRNA expression profiles of stage-matched samples during OS regeneration of *C. robusta* (Hamada *et al*., 2015; Spina *et al.,* 2017). Furthermore, in the attempt to develop cell cultures from *Ciona* larva, a simple method has been devised that allows the maintenance of dissociated neuronal cells, together with other cell types, from *Ciona* in primary culture for 2 weeks (Zanetti *et al.*, 2007). This opens the possibility that this method, further improved and refined, could be used in a wide range of experiments on this animal model, including studies of the biochemical, molecular and biophysical properties of individual cells in the larval nervous system of *Ciona*.

Effects of biological extracts on terminal differentiation of HL60 & NB4 leukaemia cell lines

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Cancer is the second leading cause of death

worldwide (22.8%) following heart disease (26.6%). Unlike normal cells, cancer cells show a block in differentiation leading to uncontrolled proliferation, eventually invading surrounding tissues and organs. This can lead to metastases as the cancer cells spread to other parts of the body through the haemopoietic and lymphatic systems. The aim of this study is to cause terminal differentiation of cancer cells using extracts from the axolotls (*Ambystoma mexicanum*), a highly regenerative organism able to regenerate complex structures. Axolotl extract (AXE) was tested against HL60 & NB4 leukaemia cell lines. AXE was also tested in combination with two histone deacetylase inhibitors namely belinostat and BML-210. Following treatment of different cell lines, AXE was evaluated for its ability to induce granulocytic differentiation using the NBT test. Its anti-proliferative and cytotoxic effects were tested using the MTT assay, and trypan blue was used to determine the number of live and dead cells. HL-60 cells were also tested for cell surface antigens CD11b and CD14, cell cycle analysis, and degree of apoptosis.

Key results: AXE had a differentiation effect on both NB4 and HL60 cells. Both cell lines exhibited granulocytic differentiation that was detected using the NBT test and CD11b surface marker.

Conclusions and implications: These results indicate that AXE merits further investigation to elucidate the pathways in which they are implicated and also isolate and identify any active compounds that can be used in cancer therapy

Cnidarian primary cell culture as a tool to investigate the effect of thermal stress at cellular level

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In the context of global change, symbiotic cnidarians are largely affected by seawater temperature elevation leading to symbiosis breakdown. This process, also called bleaching, is triggered by the dysfunction of the symbiont photosystems causing an oxidative stress and cell death to both symbiont and host cells. In our study, we wanted to elucidate the intrinsic capacity of isolated animal cells to deal with thermal stress in the absence of symbiont. In that aim, we have characterized an animal primary cell culture form regenerating tentacles of the temperate sea anemone *Anemonia viridis*. We first compared the potential of whole tissue tentacle or separated epidermal or gastrodermal monolayers as tissue sources to settle animal cell cultures. Interestingly, only isolated cells extracted from whole tentacles allowed establishing a viable and proliferative primary cell culture throughout 31 days. The analysis of the expression of tissue specific and pluripotency markers defined cultivated cells as differentiated cells with gastrodermal origin. The characterization of the animal primary cell culture allowed us to submit the obtained gastrodermal cells to hyperthermal stress (+ 5°C and + 8°C) during 1 and 7 days. Though cell viability was not affected at both hyperthermal stress conditions, cell growth drastically decreased. In addition, only a + 8°C hyperthermia induced a transient increase of antioxidant defences at 1 day but no ubiquitin or carbonylation protein damages. These results demonstrated an intrinsic resistance of cnidarian gastrodermal cells to hyperthermal stress and then confirmed the role of symbionts in the hyperthermia sensitivity leading to bleaching.

Metabarcoding of symbionts in the Mediterranean stony coral (*Cladocora caespitosa*): preliminary work

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The foundation of coral reef biology is the coral holobiont – an entire community of living organisms that make up a healthy coral head: the coral animal itself, its endosymbiotic algae or zooxanthellae (dinoflagellate genus *Symbiodinium*), and other resident microbes (bacteria, fungi and green algae). Anthropogenic disturbances, especially thermal stress, can strongly affect this dynamic symbiosis mainly by decreasing the photosynthesis/photosynthetic efficiency of the symbiotic zooxanthellae, which most often leads to their expulsion from the coral host commonly known as coral bleaching. Bleaching events are associated with nutritional depletion of the coral, its impaired reproduction and increased susceptibility to disease and mortality. While responses to thermal stress have been well investigated in tropical corals, responses of their counterparts inhabiting temperate waters is less studied. Mediterranean stony coral (*Cladocora caespitosa*) is the only coral reef building scleractinian coral living in the Mediterranean Sea, where it is spread in almost all biogeographic areas. Mass bleaching events in *C. caespitosa* colonies are observed regularly in the Adriatic since 1997 with even subsequent mass mortality in some cases. However, it appears that populations inhabiting North Adriatic could be more resistant to bleaching events. During the project "Response of biogenic formations to heat stress and the consequences for ecosystem services" funded by the Slovenian Ministry of Education, Science and Sport, the European Regional Development Fund (5442-15/2016/18 Researchers at the beginning of their careers 2.0) and the Slovenian Research Agency (P1-0237 Coastal Sea Research) shifts in the host–microbiota associations due to the thermal stress will be investigated using a common garden

experimental approach. Corals from different sources will be exposed to an increase in temperature over a longer and shorter period and the diversity of their holobiont will be assessed with a metabarcoding approach. As most suitable common metabarcoding markers have low phylogenetic resolution and can miss a large portion of the biodiversity a multi-marker approach will be used for identification of both prokaryotic and eukaryotic organisms.

Coral cell culture and their use in biomineralization study

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Coral biomineralization is important at the organismal, ecosystem, and global scales, yet the biological component has not been well understood. In particular, identities, roles, and environmental susceptibility of the proteins retained in coral skeleton were previously unknown. Understanding the cellular and molecular responses of stony corals to ocean acidification is key to predicting their ability to calcify under projected high CO_2 conditions. Of specific interest are the links between biomineralization proteins and the precipitation of new calcium carbonate ($CaCO_3$), which potentially can provide a better understanding of the biomineralization process. To address this, we developed a novel coral tissue cultures to investigate the biophysical mechanism of calcification in corals. Our goals were to (a) establish an experimental system in which calcification is facilitated at the cellular level, while simultaneously allowing in vitro manipulations of the calcifying fluid, (b) to test the effects of increased CO_2 on the calcification process at the cellular and molecular levels, and (c) to study the mineral initiation mechanism in corals.

Viable cell cultures of the hermatypic, zooxanthellate coral, *Stylophora pistillata*, have been maintained for 6 to 8 weeks. Using an enriched seawater medium with aragonite saturation state which mimiks open ocean surface waters (Ω arag ~4). We have shown that within 72 hr after isolation, cultures of separated coral cells aggregate into proto-polyps and form an extracellular organic matrix (ECM) and precipitate aragonite crystals at a rate comparable to the intact organism and with geochemical properties similar to parent skeleton.

In addition, our results suggest that compensatory molecular adjustments to deal with ocean acidification are successful only up to a point, beyond which these mechanisms cannot compete with local chemical conditions unfavorable to biomineralization. We further suggest that calcium is concentrated in intracellular pockets that are subsequently exported from the cell where a nucleation process leads to the formation of extracellular aragonite crystals.