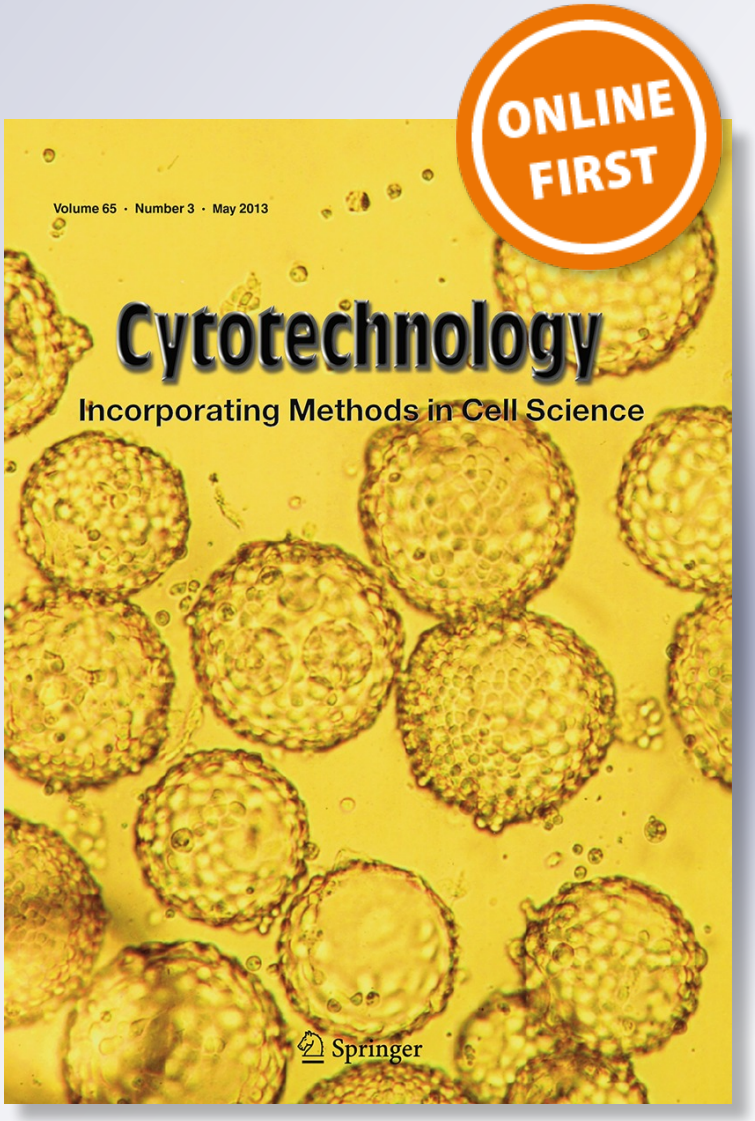
Proceedings of the symposium 'marine invertebrate cell culture'

Cytotechnology
Incorporating Methods in Cell Science
International Journal of Cell Culture and Biotechnology

ISSN 0920-9069

Cytotechnology
DOI 10.1007/s10616-013-9610-2



Springer

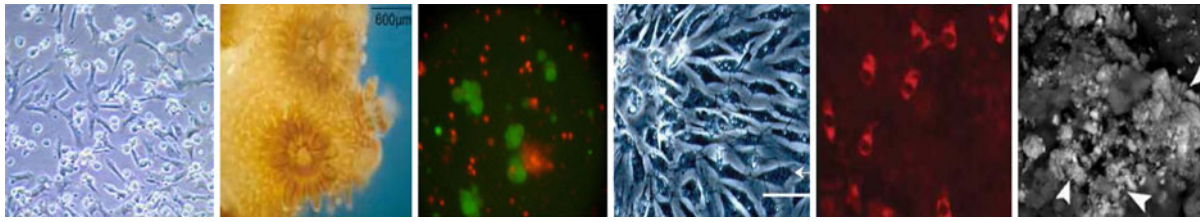
Cytotechnology
DOI 10.1007/s10616-013-9610-2

SI: MARINE INVERTEBRATE CELL CULTURE

Proceedings of the symposium 'marine invertebrate cell culture'

Concarneau, France, August 30–31 2012

Muséum national d'Histoire naturelle



Concarneau, France
August 30–31 2012

Guest Editors:
Isabelle Domart-Coulon and Stéphanie Auzoux-Bordenave
Muséum national d'Histoire naturelle

Published online: 12 July 2013

Preface

The symposium '**Marine Invertebrate Cell Culture'** was held at the 'Marine Biology Station of the French Muséum national d'Histoire naturelle, in Concarneau (France) on August 30 and 31 2012. In the context of the expanding field of marine biotechnology, this symposium aimed at promoting scientific exchanges and transfer of knowledge on marine invertebrate cell cultures. In the past 50 years, the progress in vertebrate cell culture models demonstrated the value of in vitro systems as biological models targeting specific organs or cell types. These models enabled research on fundamental biological questions such as cell lineage differentiation processes, symbiosis, biomineralization, as well as the development of in vitro models of human or other vertebrate diseases and assays for pathology and ecotoxicology. However, in contrast to vertebrate cells, the development of marine invertebrate cell cultures has progressed slowly, and in the last five decades, all attempts to produce continuously proliferating cell cultures have failed. To date no marine invertebrate cell line exists and we are still using primary cell cultures which cannot be propagated more than a few times after their establishment from the organism.

Twenty years ago, a symposium entitled 'Marine invertebrate cell culture: breaking the barriers' was held in Anaheim, California, by the National Oceanographic and Atmospheric Administration. Since then, there have been a few isolated communications reporting technological improvements or applications of marine invertebrate cell cultures, mostly in meetings of the Society for In Vitro Biology and Marine Biotechnology Conferences. This research field is very fragmented, both in space (involving a handful of scientists in academic institutions from different countries) and in time (as many scientist stop exploring this technology when they face persistent difficulties and failure). Marine invertebrate cell culture is a field requiring substantial funding (for cell culture equipment, consumables and reagents) for a slow return on investment, in terms of success and publications. Most failures are not reported, so the same mistakes are often repeated.

The present MICC symposium gathered 52 participants, senior and early-stage researchers, from 12 countries, to exchange their experiences and discuss recent advances and current challenges in the field. The main topics covered in this symposium included cell lineages and proliferation, 3D culture and tissue regeneration, cell-microorganisms interactions and various applications of primary cell cultures. Communications were distributed within five sessions of oral presentations, each introduced by a keynote presentation, and two poster sessions. The communications highlighted the great diversity of the cellular models originated from sponges, cnidarians, molluscs, crustaceans and echinoderms, including commercially important species. The symposium concluded with two round table discussions on 'Technical issues' and 'Stem cell cultures'.

This Special Issue of the journal *Cytotechnology* contains the abstracts of all the contributions presented at the symposium and 7 manuscripts peer-reviewed by external referees according to the review procedure of the journal. We wish to thank all authors who submitted abstracts and/or manuscripts for publication and referees for their care in the review process.

The symposium committee acknowledges the Muséum national d'Histoire naturelle (MNHN), the Centre National de la Recherche Scientifique (CNRS), the GIS Europôle Mer and the City of Concarneau for their support. The committee also wishes to thank the director and staff of the Station de Biologie Marine of the MNHN in Concarneau who hosted the symposium, for their help in organizing convivial and friendly breaks between sessions, thus contributing to successful informal exchanges between participants.

A consensus was reached between participants to organize regular gathering of the scientific community involved in marine invertebrate cell cultures, at few year intervals, to strengthen the development of this research field.

The symposium committee:

Dr. Stéphanie Auzoux-Bordenave (MNHN-UPMC, Concarneau, France)
Dr. Isabelle Domart-Coulon (MNHN, Paris, France)
Pr. Dominique Doumenc (MNHN, Paris, France)
Pr. Yves LeGal (MNHN, Concarneau, France)
Pr. Werner Müller (Univ Mainz, Germany)
Dr. Christine Paillard (LEMAR, Brest, France)
Pr. Shirley Pomponi (Florida Univ., USA)
Pr. Baruch Rinkevich (NIO, Haifa, Israël)

Contents

Haemocyte primary-culture from three Mollusc species and its application in ecotoxicology
Charles Le Pabic, Antoine Mottier, Katherine Costil, Noussithé Koueta, Jean-Marc Lebel, Antoine Serpentini
Development of primary cell cultures from sea urchin gonads
Silvia Mercurio, Cristiano Di Benedetto, Michela Sugni, M. Daniela Candia Carnevali
Characterization of abalone *Haliotis tuberculata–Vibrio harveyi* interactions in gill primary cultures
Delphine Pichon, Benoit Cudennec, Sylvain Huchette, Christine Paillard, Stéphanie Auzoux-Bordenave
Establishment of a primary cell culture from *Crangon crangon* and their characteristics
Frauke Symanowski, Bianka Grunow

Session 1: Cell lineages and proliferation

Cell cultures from marine invertebrates: past failures and future promises

Baruch Rinkevich

Israel Oceanographic and Limnological Research, National Institute of Oceanography, Tel- Shikmona, P.O. Box 8030, Haifa 31080, Israel

Corresponding author: Baruch Rinvevich, buki@ocean.org.il

Despite decades of extensive research efforts, and notwithstanding all attempts, immortalized cell lines from marine invertebrates are not yet available, in part, because of the wide variety of cell types in marine invertebrates from different phyla, limited knowledge on the nutritional needs, growth factors and other unique conditions that support proliferation of marine invertebrate cells in vitro. While the biological reasons of these failures are still elusive, it is customary to document that marine invertebrate cells stop dividing in vitro within 24–72 h after their isolation, starting cellular quiescence. The limited achievement in marine invertebrate cell cultures is also associated with the fact that scientific journals usually avoid publishing failed experiments, so much of the unsuccessful attempts are not presented to the scientific community. Summarising past failures in the development of cell cultures from marine invertebrates, novel biological methodologies, recently developed, bring new approaches and new hopes for successful development of cell cultures from these organisms. For example, evaluating the list of cell lines developed from insects and mammals elucidates that a significant portion of these new cell lines represents transformed cells, immortal cells acquired from naturally developed tumors, hybridomas, induced mutagenesis or plasmid transfected cells, use of adult stem cells, employment of induced pluripotent stem (iPS) cells, and additional new approaches. Also, the fast application of genomic and proteomic methodologies in marine biology, may enable researchers to survey globally the alterations at messenger RNA and protein levels for advancing the knowledge on in vitro cellular quiescence versus cell proliferation. The present talk will summary current trends and scientific approaches in the research discipline of invertebrate cell cultures and will focus on novel promising avenues in the research. It is claimed that recapturing cellular immortality that has failed when using the traditional approaches for cell cultures can be successfully achieved by employing modern molecular and cellular tools, developed for mammalian systems.

Keywords: Marine invertebrates, Cell division, Quiescence, Stem cells, Immortalization

Spicule formation and pigment cell differentiation in primary cell cultures of sea urchin embryos. Cryopreservation of the cultures

Nelly Odintsova[1,2], Natalia Ageenko[2], Andrey Boroda[1,2], Yulia Kiprushina[1,2]

[1]Far Eastern Federal University, 690950, Sukhanova Str. 8, Vladivostok, Russia; [2]A.V. Zhirmunsky Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences, 690059, Palchevsky Str. 17, Vladivostok, Russia.

Corresponding author: Nelly Odintsova, nelodin54@yahoo.com

Marine organisms passed through the long path of evolution and adaptations; this is duly reflected in the peculiarities of their biosynthesis and metabolism. The purpose of the study is to reveal exogenous factors that influence the implementation of the spicule- and pigment-formation program in a culture of sea urchin embryonic cells and to estimate the effect of these factors on cell differentiation. As shown by Okazaki (1975), isolated sea urchin micromeres can under certain conditions differentiate into cells capable of forming spicules. We have found that the process of spicule formation depends on the substrate type and the medium composition. The maximal number of spicules was detected in cells cultivated on fibronectin. For the first time, we have shown that the serum required for spicule formation in vitro can be replaced by a complex of factors, including insulin, transferrin, and lectins. Recently, we have characterized the expression of a new gene, *Si-VEGF2*, which is a member of the vascular endothelial growth factor family in the sea urchin *Strongylocentrotus intermedius*. Based on the RT-PCR and in situ hybridization results, we assume that Si-VEGF2 can play an essential role in skeleton formation. In normal development, the nonskeletogenic mesoderm gives rise to several differentiated cell types, one of which is the pigment cell type. Pigment cells could provide a source of pharmacologically important quinone pigments that would help to reduce the impact on the adult sea urchin population. We have previously shown that some foreign genes, such as the yeast transcriptional activator *gal4* gene, can be incorporated into the genome of sea urchin embryos inducing abnormal embryo development. After 20 days of cultivation of the transformed embryos, they dissociated into single pigment cells. Here we continued the studies of the pigment differentiation of sea urchin cells in culture and developed conditions for committed differentiation of pigment cells without transfection of sea urchin embryos with foreign genes. After 2–3 days of cultivation, the cells of a blastula-derived culture were transferred into new dishes with fibronectin coated coverslips. Shikimic acid, the precursor of naphthoquinone pigments, has been found to affect the expression of some pigment cell-specific genes in the cell culture. The clearest effect was detected with sea water medium and the coelomic fluid of injured sea urchins. We failed to develop a potential permanent

cell line; however, the culture conditions used can be useful for studying sea urchin pigment cells. To preserve cells in their original state we have improved a cryopreservation method for echinoderm cells. After freezing-thawing in the medium containing disaccharide trehalose, antioxidant echinochrome and total lipid extract of mussel tissues, the output of viable sea urchin cells reached 75–80 %. Our results have shown the synergistic activity of simultaneous using antioxidants and exogenous lipids as cryoprotective components. The thawed cells attached to the substrate, some synthesized pigment granules and spicules, a part of the cells aggregated to form embryo-like structures that moved actively during 5–21 days. The results of this study may be valid to solve practical problems in marine biotechnology, such as the establishment of cell cultures capable of producing mineral structures and biologically active substances. This work was supported by the Program of Far Eastern Federal University (11 G34.31.0010), Presidium of FEB RAS (12-I-0-02-027, 12-I-0-06-015, 12-III-A-06-005, 12-III-B-06-031) and RFBR (12-04-00363, 12-04-31974).

Keywords: Cell culture, Sea urchin, Embryos, Pigment differentiation, Cryopreservation

Extracellular matrix is required for muscle differentiation in primary cell culture of larval *Mytilus trossulus* (Mollusca: Bivalvia)

Vyacheslav Dyachuk

A.V. Zhirmunsky, Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences, 690059, Palchevsky St. 17, Vladivostok, Russia

Corresponding author: Vyacheslav Dyachuk, slavad83@gmail.com

Myogenesis involves the processes of cell proliferation, differentiation, migration, cell–cell interactions and development of myofibers. Components of the extracellular matrix (ECM) may modulate the growth factor effects that play an important role in myogenesis and muscle plasticity. Previously, we showed that differentiation into muscle cells occurs during cultivation of mussel cells from premyogenic larval stages and described the synthesis of specific muscle proteins and muscle fiber assembly.

In this study, we examined the interaction of cultured mussel cell with components of ECM using specific muscle antibodies. ECM proteins were found to affect the cell morphology, muscle protein synthesis and their assemblage in myofibril structures. Mussel cells grown on fibronectin or poly-D-lysine had normal bipolar cell morphology and correctly distribution of muscle proteins in cells, while cell spreading and commitment of contractile phenotype of mussel cells cultivated on collagen carpets were inhibited. The results of our experiments with RGDS-peptide, the inhibitor of integrin receptors and cell adhesion, and the control non-specific RGES-peptide suggest that cultivated mussel cells use an integrin-dependent mechanism for adhesion and outgrowth on different ECM substrates. RGDS-peptide blocked cell adhesion and inhibited myogenic differentiation, whereas incubation of the cells with RGES-peptide did not affect myodifferentiation. Finally, we began to analyze of distribution of $\alpha v\beta 3$ and $\beta 1$-subunit of integrins in primary mussel cell culture with the goal of identifying how mechanism of muscle differentiation might be modulated by integrin receptors.

Keywords: Cell culture, Mussel, Myogenic differentiation, ECM, Integrins
(This work is supported Presidium of FEB RAS, grant 12-I-0-02-027).

Germinal niche of the oyster *Crassostrea gigas*: cellular and molecular characterization

Sébastien Chong[1], Clothilde Heude Berthelin[1], Alban Franco[1], Ramdane Khider, Kristell Kellner[1], Didier Goux[2], Christophe Lelong[1]

[1] Biologie des Mollusques marins et des Ecosystèmes Associés FRE3484 BioMEA - CNRS INEE, [2] Centre de Microscopie appliquée à la Biologie SFR ICORE

Corresponding author: Christophe Lelong, christophe.lelong@unicaen.fr

The cupped oyster *Crassostrea gigas* represents the first resource of aquaculture in the world and France is the first European producer of this species. And then, the control of reproduction of this species constitutes a checkpoint to the success of mass production. Indeed, the production of spats obtained from genitors ripened under artificial conditions fails at certain periods of the year pointing out the necessity to understand and control mechanisms of gametogenesis re-initiation. To have an overview of the fine regulative mechanisms involved in gametogenesis, a precise knowledge of the tissues and the role of each cell type present in gonad are required. In many cases, intimate interactions between germinal lineage and somatic support cells are necessary for normal germ cell behaviour and differentiation. Finally, the characterization of these totipotent primordial cells may help to overlap the difficulty to obtain proliferating cells cultures required for molluscs.

Knowledge of organization and regulations inside the germinal niche at the beginning of gametogenesis is of great interest as gonial mitosis are one of the key of the success of reproduction. Cellular organization of germinal niche in oyster was studied and first assays of isolation of cell types in the niche performed. Expression of markers of early germ cells and somatic cells having a role in the niche were measured in order to specify the regulations inside the niche.

Ultrastructural organization of the niche was established and showed that the Intragonadal Somatic Cells (ISCs) associated to the germ line played a major role in the arrangement of germ lineage in gonadal tubules in the male oyster *C. gigas*. A procedure for isolation of early germ cells and ISCs based on enzymatic dissociation and density gradient was developed and allowed to obtain enriched cell fractions qualified by ultrastructural study and molecular expression of specific markers.

Keywords: Mollusc, Oyster, Reproduction, Germinal niche, Cell isolation

Subpopulation of coelomic epithelium cells of starfish *Asterias rubens* L. able to long-term proliferation in culture

Olga Petukhova, Sergey Shabelnikov, Natalia Sharlaimova

Institute of Cytology RAS, Saint-Petersburg, Russia

Corresponding author: Olga Petukhova, petukhova@yandex.ru

Echinoderms provide attractive experimental models to study the mechanisms of regeneration and stem cell biology owing to their outstanding capability to regeneration. The revealing of responsible cells is one of the main questions which needs to be resolved. Starfish regenerate at a much slower rate than other echinoderms. The immune/haematic system is the only adult tissue of *A. rubens* which is able to rapidly self-renew. In *A. rubens*, a low level of proliferation has been shown in the early period of regeneration (1). Hence, the migration of preexisted cells from some reservoir is suggested. The most probable candidate for this role seems to be the coelomic epithelium (CE) (1, 2). In our previous investigations we have developed the method of animal injury, which resulted in increasing the proportion of small cells with high nuclear-cytoplasmic ratio in the coelomic fluid in the early period post-injury (up to 7 days). The data suggested coelomocytes renewal from a stock of poorly differentiated cells (3). In coelomic epithelium the subpopulation of cells enriched with small epitheliocytes morphologically resembles small cells of the coelomic fluid, and the ability of these cell types to migrate from coelomic epithelium into the coelomic fluid has been found. The localization of these small cells within the coelomic epithelium has been characterized by means of histological analysis, whole-mount technique and scanning electron microscopy. Methods of isolation and cultivation of cells from various tissues of the sea star *A. rubens* L. were elaborated and an analysis of the behavior in culture and incorporation of BrdU was performed (4). A reproducible BrdU incorporation was detected in CE cells both in vivo and in vitro. After 2 months of cultivation, the formation of colony-like aggregates composed of small epitheliocytes with high nuclear-cytoplasmic ratio, incorporating BrdU, was characteristic of CE cells. Based on previously obtained data demonstrating the selective attachment of small epitheliocytes to laminin (5) we undertook the cultivation of CE cells on laminin and the estimation of their proliferative activity. First, laminin attachment resulted in increased proportion of cell proliferation compared to in vivo. Secondly, 5 days after seeding, small aggregates formed which were composed of small cells with high nuclear-cytoplasmic ratio, while the large coelomocyte-like cells formed networks. Later, such small cell types were found in large aggregates on the surface of well-spread cells. Thirdly, proliferative activity in vitro was confirmed via BrdU incorporation and staining with the anti-phospho-histone H3 antibody mitotic cell marker. Proliferation was preserved in culture at least up to 1 month. Fourth, two types of proliferating cells were found with different behavior in culture: either preferentially attached to substrate, forming aggregates, or small cells detached during cultivation. Morphology of small epitheliocytes, proliferative activity and ability to migration suggest that small epitheliocytes possess some properties of stem cells.

Keywords: *Asterias rubens*, Coelomic epithelium, Primary cell culture, Small epitheliocytes, Proliferative activity

The work is supported by the grant of RFBR 10-04-001-74a; grant SPb Gov. 2011.

References

Moss C, Hunter AJ, Thorndyke MC 1998. Phil. Trans. R. Soc. Lond. B 353: 421–436

Vanden Bossche JP, Jangoux M 1976. Nature. 261: 227–228.

Kozlova A, Petukhova O, Pinaev, G Tsitologiya (Russ) 2006. 48: 175–183

Sharlaimova, GP Pinaev, OA Petukhova, 2010. Cell and Tissue Biology, 2010, 4: 280–288. Original Russian Text-Tsitologiya. 2010. 52: 317–325

Sharlaimova, OA Petukhova. 2012. Cell and Tissue Biology. 6: 176–188. Original Russian Text- Tsitologiya. 2011. 53: 891–902

Session 2: 3D culture and tissue regeneration

Hydra—a simple in vivo model allowing cellular analysis at in vitro depth

Alexander Klimovich

Thomas C.G. Bosch, Zoological Institute, Christian-Albrechts University, Olshausenstrasse 40, D-24098 Kiel, Germany

Corresponding author: Alexander Klimovich, aklimovich@zoologie.uni-kiel.de

For years, cnidarians drew researcher's attention due to important phylogenetic position—as a basal Metazoan and sister group to Bilateria. Moreover, significant ecological and economical role of the corals stimulated research on this group of invertebrates. At the same time scleractinian and octocorallian corals turned out to be quite difficult to be investigated at cellular level, and up to now laboratory culture of Anthozoa is still a challenge. This limitation led an easy-to-culture freshwater hydrozoan *Hydra* to become a «lab rat» among cnidarians, and an extensive toolkit of methods was developed to use this model in research at cellular and molecular levels.

Short-term cell cultures, transplantation and reaggregation procedures, as well as different labeling techniques made possible the identification of all cell lineages in *Hydra*, which was not yet achieved in any other cnidarians. Recently emerged complete genome sequencing of *H. magnipapillata* and lineage-specific transcriptome sequencing data (www.compagen.org) provided insight into the identification of key molecules for stem cell maintenance, commitment and differentiation (Hemmrich et al. 2012). Finally, transgenesis technique became available for *Hydra*, thus offering unlimited potential for detailed analysis of gene function by stable up- or down-regulation of gene of interest (Khalturin et al. 2007; Boehm et al. 2012). Altogether, this toolkit allows addressing in *Hydra* model plenty of questions, which are currently being under investigation worldwide. The evolution of signaling networks and patterning processes in development, the complexity of stem cell regulation and cell fate determination, the evolution of innate immunity and host-microbe interactions—that is just a bare list of question in focus

of research today. In applied biotechnology, *Hydra* took its place as well—as a novel source of potential bioactive molecules (e.g. antimicrobial peptides) and a bioreactor for cheap production of recombinant proteins.

In summary, *Hydra*, being a relatively simple model organism, allows performing in vivo cellular analysis at a level comparable with that of in vitro cell culture-based systems.

Keywords: Hydra, Model, Molecular analysis

References

Hemmrich G, Khalturin K, Boehm AM, Puchert M, Anton-Erxleben F, Wittlieb J, Klostermeier UC, Rosenstiel P, Oberg HH, Domazet-Lošo T, Sugimoto T, Niwa H, and Bosch TCG (2012) Molecular signatures of the three stem cell lineages in Hydra and the emergence of stem cell function at the base of multicellularity. Mol Biol Evol. 29(11):3267–3280.

Khalturin K, Anton-Erxleben F, Milde S, Plötz C, Wittlieb J, Hemmrich G and Bosch TCG (2007) Transgenic stem cells in Hydra reveal an early evolutionary origin for key elements controlling self-renewal and differentiation. Dev. Biol. 309: 32–44

Boehm AM, G Hemmrich, K Khalturin, M Puchert, F Anton-Erxleben, J Wittlieb, UC Klostermeier, P Rosenstiel, H-H Oberg, and TCG Bosch (2012) FoxO is a critical regulator of stem cell maintenance and immortality in Hydra. Proc. Natl. Acad. Sci. USA, in press

New perspectives in cnidarian cell culture: the model of octocoral

Carla Huete-Stauffer[1], Laura Stagnaro[1], Laura Valisano[1], Carlo Cerrano[2]

[1]DIP.TE.RIS. University of Genoa, Corso Europa 26, 16132, Italy, [2] DI.S.V.A. Università Politecnica delle Marche, Via Brecce Bianche, 60131, Italy

Corresponding author: Carla Grace Huete-Stauffer, chuetestauffer@virgilio.it

The limited knowledge in developmental features, reproduction cycles and evolutionary traits of lower metazoans as marine cnidarians compared to more evolved ones, has been in many cases related to the difficulty of maintaining cell cultures in laboratory conditions. We have tested for gorgonians a method usually applied for Porifera, leading to the production of primmorphs, which are a cell re-aggregation composed by a high abundance of stem cells. Here six Mediterranean gorgonians (*Paramuricea clavata, Eunicella singularis, E. cavolinii, E. verrucosa, Corallium rubrum* and *Lophogorgia sarmentosa*) have been treated for 'primmorphs' production.

'Primmorph' conditions of formation, number formed and maintained in the lab, BrdU incorporation tests, calcein labeling of cells and sclerite formation have been achieved in this work. The BrdU tests showed that cells were actively dividing, calcein assays and epifluorescence imaging demonstrated the presence of active carbon ions in the cells confirming that it is possible to obtain cell cultures but with different efficiency depending on the species. The best results have been obtained from *E. singularis*. The average size of formed 'primmorphs' was 0.245 ± 0.086 mm SE with a minimum size of 0.1 and a maximum size of 0.415 mm, zooxanthellae were maintained throughout the whole time frame. The possibility to obtain sclerite formation opens a wide field of investigation on in vitro biomineralization, since sclerite formation starts after about 1 month.

The application potential of gorgonian 'primmorphs' is very broad, ranging from developmental and evolutionary sciences to studying metabolic pathways of cell cycle and differentiation, use in biotechnology of second metabolites, cell cultures for environmental research and experiments on the response to stress factors or diseases, and the study of calcification rates in relation to acidification and temperature increase in a climate change context.

Keywords: Cnidarian cell culture, Primmorphs, Biomineralization, BrDU, Calcein, Sclerites

Coral cell proliferation in situ (polyp) and in primary cultures (3D aggregates)

Agathe Lecointe[1], Marc Geze[2], Shakib Djediat[2], Isabelle Domart-Coulon[1]

[1]UMR 7208 BOREA, Dpt. MPA 43 rue Cuvier Museum National d'Histoire Naturelle, Paris, France, [2] UMR 7245 MCAM, Dpt. RDDM, Centre de microscopie et d'imagerie (CEMIM) et Plate-forme de Microscopie Electronique du MNHN, Muséum National d'Histoire Naturelle, Paris, France

Corresponding author: Isabelle Domart-Coulon, icoulon@mnhn.fr

In colonial Scleractinian corals, continuous growth with periodic upward withdrawal of the tissue results in continuous vertical extension of the calcified exoskeleton, building the framework of reef ecosystems. Tissue homeostasis is controlled within the individual polyps, maintaining a balance between cell proliferation for self-renewal, and differentiation into specialized cell types. Localization of active zones of proliferation within the polyp tissue layers and in derived primary cell cultures has not yet been determined.

In this study, spatial variations of proliferation in the coral *Pocillopora damicornis* (Linnaeus 1758) have been characterized with a method based on DNA synthesis assessment via 5-bromo-2′-deoxyuridine (BrdU) incorporation into nuclear DNA over a 24 h labeling period and detection by immunolocalization with a fluorescent secondary antibody. *In situ,* BrdU was incorporated in all four cellular layers of the polyp, with lowest incorporation in the calicoderm (<5 % BrdU-positive cells) involved in skeletal formation, and highest incorporation in the gastroderm (~20–30 % BrdU-positive cells) lining the gastric cavity. An intermediate rate (~10 % BrdU-positive cells) was observed in the oral pseudo-stratified epithelium, in contact with seawater, which is the site of insertion of terminally differentiated mucocytes and cnidocytes.

In vitro, spatially heterogeneous proliferation events were recorded in tissue balls (~7 % BrdU-positive cells) which are cell aggregates with a smooth surface, formed during the second day of coral primary cultures. Furthermore a transient rise in zooxanthellae density within isolated gastrodermal host cells indicated perturbation in the control of their cell cycle upon tissue dissociation in primary culture.

Keywords: Scleractinian coral, Polyp, Cell proliferation, BrdU, Tissue dissociation, Cell aggregation

Developing coral explants and micropropagates for biotechnological uses

Esti Kramarsky-Winter

Proreef Ltd. Rehovot Israel, and Dept. of Zoology George S Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv Israel 69978

Corresponding author: Esti Kramarsky-Winter, esti.winter@gmail.com

As the marine environment is becoming increasingly affected by anthropogenically driven stressors, the development of cell and tissue models of key invertebrates is essential. Recently we harnessed the developmental plasticity of some scleractinian corals and established a process of tissue explantation and micropropagation under controlled laboratory conditions. This enabled us to produce lines of clonally derived coral tissue micropropagates, 1–5 mm in size, devoid of, or including skeletal element, from a variety of coral species, and for a variety of uses. We found that explantation of these minute tissue fragments from adult coral colonies or polyps, and maintaining them under specific and properly controlled conditions of temperature, light, salinity, and water motion regimes, gives rise to micropropagates that can either be maintained as undifferentiated tissue balls or caused to develop into polyps or colonies. Using histology, immunohistochemistry and transmission electron microscopy we followed cellular processes occurring during reorganization of the tissues following explantation. These processes include programmed cell death, cell migration, proliferation and de novo differentiation, and are comparative to processes occurring during coral tissue regeneration. We further showed that under proper conditions the explants re-differentiate into fully functioning miniature polyps that are able to feed and calcify. We then applied different physical and chemical stressors to the micropropagates, and recorded morphological and physiological changes, thus providing proof that they can be used as models for research purposes. The miniature size and ease of manipulation of these micropropagates allows the production and long-term maintenance of these clonally derived lines for numerous research purposes. In addition, repetitive explantation of micropropagates, facilitates the development of lines of specific genotypes making them amenable to comparative and collaborative studies worldwide.

Keywords: Coral, tissue explant, De novo differentiation

Session 3: Cellular models for biomineralisation and nanomaterial studies

Genetic: cell biological—structural aspects of biomineralization: Sponge biosilica formation an exceptional model

Werner E.G. Müller

ERC Advanced Investigator Grant Research Group at Institute for Physiological Chemistry, University Medical Center of the Johannes Gutenberg University Mainz, Duesbergweg 6, D-55128 Mainz, Germany

Corresponding author: Werner Müller, wmueller@uni-mainz.de

Biomineralization processes are characterized by controlled deposition of inorganic polymers/minerals mediated by functional groups linked to organic templates. One metazoan taxon, the siliceous sponges (phylum Porifera: classes of Demospongiae and Hexactinellida), have utilized these principles and even gained the property to form these polymers/minerals by an enzymatic mechanism using the silicateins. Silicateins are the dominant protein species present in the axial filament, which is the enzyme as well as the template of biomineralization, of the skeletal elements of the siliceous sponges, the spicules. Silicateins also present as a major part of the organic components in the silica lamellae which are cylindrically arranged around the axial canal, and exists also as a 33 kDa precursor in the extra-spicular space. In the spicules, only the processed, functionally active enzymes with sizes of 24–30 kDa are found. cDNAs coding for these enzymes, which belong to the cathepsin family of proteases, have been cloned both from demosponges and deep-sea hexactinellids. The proteins which are biocatalytically active when expressed in a recombinant way have been attributed to bio-silica formation in nature that proceeds at orthosilicate concentrations significantly lower than those required for silica synthesis in sol–gel chemistry. Using the demosponge *Suberites domuncula* as a model, quantitative enzymatic studies revealed that the native as well as the recombinant enzyme displays in vitro (almost) the same bio-silica forming activity as the enzyme involved in spicule formation in vivo. Monomeric silicatein molecules assemble to filaments via fractal intermediates which are stabilized by the silicatein-interacting protein, silintaphin-1. This silicatein interactor associates in a 1:4 stoichiometric ratio with silicatein, and thereby augments the enzymatic activity of the protein. Besides of the silicateins, and complementing those anabolic enzymes, a silica-degrading silicase acting as a catabolic enzyme has been identified. Growth of spicules proceeds in vivo in two directions. First, by axial growth, a process that is controlled by evagination of cell protrusions and mediated by the axial filament-associated silicateins. And second, by appositional growth that is driven by the extra-spicular silicateins, a process that provides the spicules with the final size and morphology. This radial layer-by-layer accretion is directed by organic cylinders which are formed around the growing spicule and consist of galectin and silicatein. Within those cylinders the siliceous lamellae are formed. Ca^{2+} ions which are required for the assembly of the silicatein/galectin cylinders are supplied by the Ca^{2+}-binding protein, silintaphin-2. The cellular interplay that controls these morphogenetic processes during spiculogenesis is outlined.

Keywords: Biomineralization; Biosilica; Fractals; Sclerocytes; Silicatein; Siliceous spicule; Silintaphin; Sponges; Suberites domuncula; Syneresis

The relationship among cell, organic matrix and biomineralization in freshwater pearl oyster

Yufei Ma, Qingling Feng

Department of Materials Science and Engineering, Tsinghua University, Beijing 100084, China

Corresponding author: Qingling Feng, biomater@mail.tsinghua.edu.cn

Hyriopsis cumingii (Zhejiang province, China) is the most widely used animal for freshwater pearl farming in China. Pearl is a kind of common biomineral produced from the biomineralization process. The inorganic component of the pearls is constituted by aragonite crystals regarded as aragonite pearls. While some pearls that have vaterite crystals as inorganic component are viewed as vaterite pearls (Qiao et al. 2007, 2008). *Hyriopsis cumingii* shell nacre, aragonite pearl and vaterite pearl were chosen to study the relationship among the cell, organic matrix and biomineralization.

Part of mantle in the shell, in contact with shell edge, was dissected to make a primary cell culture. The cells could migrate away from the tissue after 24 h, leading to form a clear boundary of the tissue and the cells. Aragonite crystals with different morphologies were found in the in vitro mineralization with the mantle tissue and the cells.

Water soluble matrix (WSM), acid soluble matrix (ASM) and acid insoluble matrix (AIM) from the shell nacre, aragonite pearl and vaterite pearl were extracted and biochemical analysis of these organic matrices involved in crystal formation and polymorph selection was carried out. Amino acid composition confirmed hydrophobic residues as major components of all extracts, but it revealed an imbalance in the proportion of acidic residues in WSM versus ASM and in aragonite versus vaterite. Electrophoresis gave evidence for signatures of proteins, with a 140 kDa material specific for aragonite in WSM. Conversely all ASM extracts revealed the presence of about 55 kDa components, including a discrete band in vaterite extract. The extracted soluble organic matrices were used in the in vitro mineralization assays to study the mechanism of organic matrix mediated biomineralization. WSM of aragonite pearl induces aragonite crystals, and ASM of vaterite pearl mediates the formation of vaterite crystals.

Keywords: Fresh water pearl oyster, Cell, organic matrix, Biomineralization

References

Qiao L, Feng QL, Li Z (2007) Special vaterite found in freshwater lackluster pearls. Cryst Growth Des 7: 275–279

Qiao L, Feng QL, Liu Y (2008) A novel bio-vaterite in freshwater pearls with high thermal stability and low dissolubility. Mater Lett 62: 1793–1796

Expression of biomineralisation genes in tissues and cultured cells of the abalone, *Haliotis tuberculata*

Matthew O'Neill[1,2], Béatrice Gaume[1], Françoise Denis[1,3], Stéphanie Auzoux-Bordenave[1,4]

[1]UMR BOREA 7208 CNRS/MNHN/IRD/UPMC, Muséum National d'Histoire Naturelle, Station de Biologie Marine, 29900 Concarneau, France, [2]Keele University, Keele, Staffordshire ST5 5BG, UK, [3]Université du Maine, 72085 Le Mans, France, [4] Université Pierre et Marie Curie, 4 place Jussieu, 75005 Paris, France

Corresponding author: Stéphanie Auzoux-Bordenave, bordenav@mnhn.fr

Mollusc shell biomineralisation involves a variety of organic macromolecules (matrix proteins and enzymes) that control $CaCO_3$ deposition, growth of crystals, the selection of polymorph, and the microstructure of the shell. Since the mantle and the hemocytes play an important role in the control of shell formation, primary cell cultures have been developed to study the expression of three biomineralisation genes recently identified in the abalone *Haliotis tuberculata*: a matrix protein, Lustrin A, and two carbonic anhydrase enzymes.

Mantle cells and hemocytes were successfully maintained in primary cultures and were evaluated for their viability and proliferation over time using semi-automated assay (XTT). PCR and gel photograph analysis were used to semi-quantify the gene expression and compare the level of expression in native tissues and cultured cells. The results demonstrated that the three genes of interest were being expressed in abalone tissues, with expression highest in the mantle and much lower in the hemocytes and the gills. Biomineralisation genes were also expressed significantly in mantle cells, confirming that primary cultures of target tissues are suitable models for in vitro investigation of matrix protein secretion.

This study shows that primary cultures of target tissues are suitable models to study the cellular and molecular processes of biomineralisation. Further characterization of cells and cell typing would help specify the respective roles of epithelial and circulating cells in matrix components secretion and $CaCO_3$ deposition. Experiments are also underway to investigate the production of calcium carbonate deposits in primary cultures.

Keywords: Biomineralisation, Gene expression, Primary culture, *Haliotis tuberculata*

Bio-silica and bio-polyphosphate: applications in biomedicine (bone formation)

Xiaohong Wang

ERC Advanced Investigator Grant Research Group at Institute for Physiological Chemistry, University Medical Center of the Johannes Gutenberg University Mainz, Duesbergweg 6, D-55128 Mainz, Germany

Corresponding author: Xiaohong Wang, wang013@uni-mainz.de

Bio-silica represents the main mineral component of the siliceous sponge skeletal elements (spicules), while bio-polyphosphate (bio-polyP), a multifunctional polymer existing in microorganisms and animals, acts, among others, as reinforcement for pores in cell membranes. These natural inorganic biopolymers which can be readily prepared, either by recombinant enzymes (bio-silica and bio-polyP) or chemically (polyP), are promising materials/substances for the amelioration and/or treatment of human bone diseases and dysfunctions. It has been demonstrated that bio-silica causes in vitro a differential effect on the expression of the genes *OPG* and *RANKL*, encoding two mediators that control the tuned interaction of the anabolic (osteoblasts) and catabolic (osteoclasts) pathways in human bone cells. Since bio-silica and bio-polyP also induce the expression of the key mediator *BMP2* which directs the differentiation of bone-forming progenitor cells to mature osteoblasts and in parallel inhibits the function of osteoclasts, they are also promising candidates for treatment of osteoporosis.

Keywords: Bio-silica; Bio-polyphosphate; Sponges; Hydroxyapatite; Bone biomaterials; Osteoporosis.

Silicatein interactors and structure-guided biosilica formation

Heinz C. Schröder

ERC Advanced Investigator Grant Research Group at Institute for Physiological Chemistry, University Medical Center of the Johannes Gutenberg University Mainz, Duesbergweg 6, D-55128 Mainz, Germany

Corresponding author: Heinz C. Schröder, hschroed@uni-mainz.de

The formation of the siliceous sponge spicules involves the formation of an organic cylinder-like structure in the extraspicular space, containing the enzyme silicatein and a calcium-dependent lectin. Applying yeast two-hybrid library screening and a newly developed solid-phase pull-down assay, we discovered, in addition to silicatein, two silicatein-associated proteins that contribute to spicule formation: (i) Silintaphin-1 a molecule that is involved in the shape-determination of the polymeric silica synthesized by silicatein; and (ii) silintaphin-2, a Ca^{2+}-binding protein. Silintaphin-2 is processed from a longer-sized 15-kDa precursor to a truncated, shorter-sized 13 kDa calcium-binding protein via proteolytic cleavage, mediated by the bone morphogenetic protein-1 (BMP-1). In primmorphs from *Suberites domuncula* retinoic acid causes a strong up-regulation of the expression of the gene encoding BMP-1. The expression levels of silicatein-α and silintaphin-2, which are strongly increased in the presence of silicate, are not affected by retinoic acid. More important, immunogold electron microscopy revealed that only in the presence of both silicate and retinoic acid the organic cylinder is formed, that surrounds the spicules and allows the radial apposition of new silica layers and hence the growth of the spicules. We conclude that retinoic acid regulates the formation of the organic cylinder. The two key enzymes of the retinoid pathway, the β-carotene dioxygenase and the retinal dehydrogenase have been *clo*ned from *S. domuncula*. Based on these results, we propose a new scheme for spicule formation.

Keywords: Biosilica; Siliceous sponges; Spicules; Silicatein; Silintaphin-1 and -2; BMB-1; Retinoic acid

Session 4: Cell: microorganisms interactions, cellular models for pathology

Hematopoietic stem cell culture in crayfish as a model for studies of hemocyte differentiation and immunity

Irene Söderhäll

Department of Comparative Physiology, Evolutionary Biology Centre, Uppsala University, Norbyvägen18A, 752 36 Uppsala, Sweden

Corresponding author: Irene Söderhäll, irene.soderhall@ebc.uu.se

Hematopoiesis is the process by which blood cells (hemocytes) mature and subsequently enter the circulation and we have developed a new technique to culture the hematopoietic progenitor cells in vitro. The reason for the successful culture was the isolation of a plasma protein that turned out to be a novel cytokine, astakine 1 (Ast1) containing a domain present in several vertebrates, so-called prokineticins. Now we have detected several astakines from other invertebrate species. Depending on our discovery of the cytokine Ast1 we have an opportunity to study in detail the differentiation of cells in the hematopoietic tissue (Hpt) of a crustacean, a tissue of evolutionary interest for studies of the connection between the vascular system and the nervous system. We have been able to isolate the entire hematopoietic tissue and for the first time detected a link between this tissue and the brain. We have further localized a proliferation center (APC) in the tissue and characterized its different parts. We have also used this system to isolate a new hematopoietic factor CHF, a small cysteine rich protein with high similarity to the N-terminal region of vertebrate CRIM1 that is important in the crossroad between apoptosis and hemocyte differentiation. The formation and development of hemocytes involve proliferation, commitment and differentiation from undifferentiated hematopoietic cells. Our technique for culture of crayfish hematopoietic stem cells provides a simple tool for studying the mechanism of astakine induced hematopoiesis, but also enables detailed studies of immune defence reactions. Further, the culture system has been used for studies of viral defence and the system is suitable for gene silencing which allow functional characterization of different molecules involved in host defence as well as in hemocyte differentiation.

Keywords: Hematopoiesis, Crustacean, Astakine 1, Hemocyte differentiation, Immune defense

Development of two reproducible haemocyte culture systems for application in crustacean immunity studies

João Dantas-Lima[1], Mathias Corteel[1], Dang Oanh[2], Peter Bossier[3], Patrick Sorgeloos[3], Hans Nauwynck[1]

[1] Faculty of Veterinary Medicine, Department of Virology, Parasitology and Immunology, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium, [2] Department of Aquatic Biology and Pathology, College of Aquaculture and Fisheries, Cantho University, Campus 2, 3-2 Street, Ninh Kieu District, Cantho City, Vietnam, [3] Laboratory of Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering - Department of Animal Production, Ghent University, Rozier 44, B-9000 Ghent, Belgium

Corresponding author: João Lima, joao.lima@ugent.be

For studying shrimp immunity, in vitro haemocyte cultures are essential. Despite the considerable amount of work that has been done in this field, well-described and reproducible culture techniques are lacking. The current work aimed to establish two in vitro culture systems for haemocytes of *Penaeus vannamei*, with cells either in attachment or in suspension, using Nunc® Nunclon™Δ Surface and Nunc® Hydrocell Surface cell culture plates, respectively. Quantified haemocyte suspensions were seeded in modified L-15 (Leibovitz) medium. Furthermore, the survival performance of haemocytes was evaluated in medium supplemented with L-glutathione (GSH) and EDTA-free protease inhibitor cocktail.

Haemocytes cultured in attachment for 1 h could be separated in adherent and non-adherent cell fractions. For the first time, attachment of shrimp haemocytes to the cell culture

substrate was successfully prevented by the Nunc® Hydrocell Surface. The clustering of haemocytes kept in suspension was recorded by cell live imaging. Haemocytes cultured under both systems could be kept up to 5 days. Supplementation with GSH significantly improved the cell survival and delayed formation and melanisation of clusters. On the other hand, addition of protease inhibitors did not improve cell survival.

In order to prove the suitability of these models for the in vitro study of shrimp immunity, the phagocytic and antibacterial activities of adherent haemocytes towards *V. campbellii* were evaluated after 1 h of co-culture. Phagocytosis was detected in 11.5 ± 0.14 % of haemocytes, with an average of 2.4 ± 0.1 bacteria per haemocyte. Furthermore, haemocytes clearly demonstrated an antibacterial activity.

It was concluded that these models could keep haemocytes functionally active during the time required for the study of innate immune responses of shrimp towards pathogens in a reproducible way.

Keywords: Crustacean immunity; Haemocyte cultures; *Penaeus vannamei*

Expression of an NFκB homologue in cultured haemocytes from the South African abalone *Haliotis midae*

Roslyn M. Ray, Vernon E. Coyne

Department of Molecular and Cell Biology, University of Cape Town, Rondebosch, 7700, South Africa

Corresponding author: Vernon E. Coyne: vernon.coyne@uct.ac.za

Haliotis midae is an important marine gastropod that is commercially farmed in South Africa. Mass mortalities and loss of production due to infectious diseases are a constant threat to abalone farmers. Consequently, a monitoring system for assessing the health status of farmed abalone is vital for effective disease mitigation. This study investigated whether nuclear factor κB (NFκB), a potential biomarker of bacterial infection of abalone, is differentially expressed in primary cultured *H. midae* haemocytes exposed to a heat killed bacterium.

NFκB is a transcription factor that participates in processes such as cell proliferation, apoptosis and the immune response. In invertebrate innate immunity, NFκB homologues regulate the activation of anti-microbial peptides in response to Gram-negative and Gram-positive bacterial, fungal and yeast infections. Two amplified fragments of an NFκB homologue were identified in *H. midae* and found to be closely related to the *Haliotis diversicolor supertexta* NFκB homologue. An in vitro 'challenge' experiment was performed to assess the response of haemocytes to heat killed FITC labelled *Vibrio anguilarium* 5676. In comparison to a control haemocyte group, phagocytic activity of the *V. anguilarium* treated haemocytes increased significantly from 2 hpi, while NFκB mRNA levels were significantly up-regulated at 6 hpi.

It was concluded that cultured haemocytes will be a useful tool for studying aspects of the *H. midae* immune response and that NFκB has potential as a biomarker of infection in farmed *H. midae*.

Keywords: Abalone; *Haliotis midae;* Nuclear factor κB

Cell tracking and velocimetric parameters analysis as an approach to assess activity of mussel hemocytes in vitro

Damien Rioult[1], Jean-Marc Lebel[2], Frank Le Foll[1]

[1] Laboratory of Ecotoxicology EA 3222, University of Le Havre, 76058 Le Havre cedex France. [2] Physiology and Ecophysiology of Marine Molluscs, UMR 100 IFREMER-UCBN, University of Caen

Corresponding author: Damien Rioult, damien.rioult@univ-lehavre.fr

Sessile bivalves belonging to *Mytilidae* are filter feeders characterized by a relatively high tolerance to environmental changes and considered as bioindicator species in many environmental studies. Immune parameters are often proposed as ecotoxicological biomarkers. In invertebrates as in vertebrates, innate immunity relies on humoral responses based on the activity of antimicrobial peptides (such as defensins) and encapsulation of microorganisms by melanization (prophenoloxidase pathway). All these proteins are secreted by the formed elements of hemolymph, collectively named hemocytes. Hemocytes are also responsible for a cell-mediated innate immunity that corresponds to cytotoxic activity (chlorination through ClO-ion production by myeloperoxidase activity) and phagocytosis. Hemocytes are found (and collected) as cells in suspension in circulating hemolymph. Hemocytes are adherent cells as well, infiltrating tissues and migrating to infected areas.

Migration activity of hemocytes remains poorly studied. However, this activity could be considered as a valuable indicator of immunocompetence and, potentially, as a biomarker of immunotoxic damage caused by exposure to environmental contaminants. Motility is related to dynamic cytoskeletal rearrangements and is controlled by external signals from endogenous or bacterial origin.

In order to study potential alterations in the motility of *Mytilus edulis* hemocytes, we have developed a method of long term cell tracking in vitro. After staining of nuclei with Hoechst 33342, we are able to monitor cell movements over periods encompassing several days and to measuring the instantaneous speeds of more than 20 cells simultaneously. Our results show that velocimetric performances of hemocytes are sensitive to the physicochemical parameters such as temperature and composition of culture media. It seems very likely that the motile activity of hemocytes reflects environmental quality.

Using this method we were able to determine the best culture conditions and thus to maintain hemocyte viability and motility over at least 4–7 days. Exposure to various environmental contaminants and comparisons between a "clean" site (Yport −76) and a site impacted by environmental contaminants (Le Havre −76) are planned to propose this method as a useful biomarker for environmental diagnostics.

Keywords: Motility, Primary cultures, Innate immunity, *Mytilus edulis*

Session 5: Cell–microorganisms interactions, cellular models for symbiosis

Sponge cell culture state-of-the-art: what's worked, what hasn't, what's next

Shirley A. Pomponi[1, 2]

[1]Harbor Branch Oceanographic Institute - Florida Atlantic University, Fort Pierce, Florida USA. [2]Wageningen University, Bioprocess Engineering Group, Wageningen, Netherlands.

Corresponding author: Shirley Pomponi, spomponi@hboi.fau.edu

Although sponge cell and developmental biology have been studied for more than 100 years, and research focused on the establishment of sponge cell lines has been conducted in earnest for at least the past 25 years, there are still no sponge cell lines. Contamination, cell dissociation techniques, and inadequate nutrient media contribute to failed attempts at establishment of sponge cell lines. Regardless, much has been learned about sponge cell and molecular biology using primary cultures. The results–both positive and negative-form the basis for the next generation of sponge cell culture research.

Cell cycling studies demonstrate that there are significant differences among species, which must be considered when selecting a model sponge (Schippers et al. 2011). Research on sponge-microbial symbioses is providing insights into the relative importance of microbes in sponge cell cultures and could guide future efforts at control of "contaminants", which perhaps should be focused more on regulating, rather than eliminating, bacteria and fungi. And molecular research, including transfection (Schippers et al. 2012), analyses of sponge genomes and transcriptomes (Riesgo et al. 2012), and gene expression studies, all provide information that will improve our ability to establish both normal and transformed cell lines.

Sponge cell cultures have the potential to be unique metazoan models for research in many diverse disciplines, including developmental biology, chemical ecology, human health, marine biotechnology, and impacts of climate change. It is important to remember, however, that there is no single protocol that works for each species: every species is different and may respond differently. Moreover, a cell line may not be required to answer the scientific questions that are being addressed; and both successes and failures generate new and unanticipated hypotheses to test.

References

Riesgo, A; Andrade, SC; Sharma, PP; Novo, M, Pérez-Porro, AR; Vahtera, V; González, VL; Kawauchi, GY; Giribet, G. Comparative description of ten transcriptomes of newly sequenced invertebrates and efficiency estimation of genomic sampling in non-model taxa. *Frontiers in Zoology* **2012a**, 9, 33. doi: 10.1186/1742-9994-9-33

Schippers KJ, Martens DE, Pomponi SA, Wijffels RH. (2011). Cell cycle analysis of primary sponge cell cultures. *In Vitro Cellular & Developmental Biology - Animal*, **47**: 302–311

Schippers KJ, Sipkema D, Osinga R, Smidt H, Pomponi SA, Martens DE, Wijffels RH (2012). Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. *Advances in Marine Biology*, **62**: 273–337

A 3D-sponge cell culture to study the pre-requisites of the installation of a bacterial symbiosis

Gaël Le Pennec

Laboratoire de Biotechnologie et de Chimie Marines, Université de Bretagne Sud-UEB, IUEM, BP92116, 56321-Lorient Cedex, France.

Corresponding author: Gaël Le Pennec, gael.le-pennec@univ-ubs.fr

Since 700 million years, sponges and bacteria co-evolve to form a symbiotic community sensu De Bary (1). Nevertheless rare studies mention the interactions between partners although molecular communications may occur to maintain a balanced association. In particular, during the early stages of the symbiosis, when one has to be recognized by the other prior to its installation e.g. during the recruitment and selection steps of bacteria by the sponge, a cross-dialogue has to be set up. Also, upstream to a physical contact, molecules such as autoinducers may circulate to inform the sponge of the presence of bacteria and to be recognized as a potential symbiot or in defect considered as nutrient. To understand how the demosponge *Suberites domuncula* reacts face to bacteria we contaminated sponge cell cultures with different bacterial elements: crude culture supernatants, a N-Acyl homoserine lactone (3-oxo-C_{12}-HSL), alive bacteria, and pure lipopolysaccharides. To achieve these experiments two bacteria previously isolated from *S. domuncula* were used: an opportunistic/pathogenic one: *Pseudoalteromonas* sp. *1A1* and a commensal one: *Endozoicomonas* sp. *Hex311* isolated on media supplemented with sponge extracts and which belongs to a genus already associated with an other sponge (2), a coral (3) and a mollusc (4). An axenic 3D-cell culture called primmorph was used to determine how the sponge behaved towards those bacteria. Preliminary results, pointing out the expression of apoptosis and immune genes, depended on the fate of the bacterium and of the bacterial products used. Mainly, a molecule implicated in the recognition pathway of bacteria, TRAF-6 and the antibacterial perforin gene (MPEG) were down regulated in presence of the living symbiotic bacterium or of its LPS while its exoproducts stimulated the immune recognition and down regulate the expression of the MPEG gene. The opportunist *Pseudomonas* sp. *1A1* or its LPS stimulate the immune recognition and perforin while its exoproducts down regulated them. Furthermore, in presence of the symbiotic bacterium the caspase gene was down regulated as well as with the opportunistic one. This led to a conclusion that the symbiotic Hex 311 was tolerated of the host immune system and prevented cell from apoptosis. The opportunist/pathogen 1A1 seemed to take the control the immune system while preventing the cell from the apoptosis so, controlling the sponge cell for its own purposes. Besides 2D-proteomic studies concerning the membrane of the primmorphs revealed that when they were cultivated in presence of the 3-oxo-C_{12} -HSL they predisposed to an endocytosis of the bacterium without presuming of the final end-point: a phagocytosis or the installation in the host cell.

The interest of the co-cultivation of eukaryotic and prokaryotic cells must be questioned in order to improve the sponge cell cultures systems.

Keywords: 3D-cell culture, Sponge, Symbiosis, Innate immunity, Apoptosis

References

1: De Bary A 1879. Die Erscheinung der Symbiose
2: Nishijima et al. 2012. Int. J. Syst. Evol. Microbiol. In press
3: Yang et al. 2010. Int. J. Syst. Evol. Microbiol. 60: 1158–1162
4: Kurahashi & Yokota 2007. Syst. Appl. Microbiol. 30: 202–206

The use of cultivated tissue fragments for in vitro experiments

Miralda Blinova, Marija Daugavet, Danila Bobkov, George Pinaev

Institute of Cytology of Russian Academy of Sciences, Tikhorezkiy av, 4, Saint-Petersburg, 194064, Russia

Corresponding author: Miralda Blinova, mira.blinova@mail.ru

Experiments were performed using sea stars *Asterias rubens* and molluscs *Mytulis edulis* from the White Sea marine biological station Kartesh. Marine invertebrates are interesting and convenient models to study regeneration process. Previously we found that sea star coelomocyte population could be restored in 5 h following their total removal due to experimental injury, resulting in high quantities compared to the initial cell population. The methods of coelomocyte net formation and net contraction from wounded sea star were developed as a model of clotting. Besides the method of wounded sea star, in vitro culture of coelomic epithelium fragments was developed. Experiments aimed at studying interactions of coelomocytes with other cell types involved in the wounding process, for instance with the coelomic epithelium cells. Epithelium fragments (explants) from wounded sea star were cultured in L-15 medium supplemented with 2 % fetal serum and placed to adhere onto the bottom of 96-well plates at 16 °C. Once the explants were attached, coelomocyte suspension from wounded sea star was introduced into the wells. After 20 min of contact, Ca^{++} solution was added in each well at 5 mM final concentration. In these conditions, coelomocytes formed a network that diplayed contractile activity in vitro. Our results show that in case of wounding, close interactions are maintained in vitro between coelomocytes and the coelomic epithelium.

We also observed very active cell migration from the sea star epithelium fragments. Some cells settled and attached to the plate surface while other cells remained in suspension. After 7–10 days of culture, migrated cells joined together and formed different tissue-like structures. Since these structures formed spontaneously in our in vitro conditions, we can suggest that explant cultures maintained cell-to-cell interactions and the synthesis of extracellular matrix proteins which are necessary to obtain tissue regeneration.

In a separate set of experiments with *Mytilus edulis* mantle fragments cultured in the same medium at 10 °C, the cells continuously migrated from the fragments. They settled, attached to surface of plate and proliferated (the number of cells increased). Some of these cells moved into suspension. Such detached cells were plated into other wells where cells again attached and proliferated. In some cases, attached cells were detached by enzymes and placed into other wells. The procedures for adherent cell passage were carried out twice, the procedure for suspended cell passage were done several times. Every time we observed formation of a new monolayer. The total time of mussel fragment cultivation was 22 months. During all time, new events of cell migration were observed. Obtained results confirm the viability of cells and cultivated fragments (explants). The formation of crystals that appeared during cultivation may also indicate the functional condition of cells. All cells and fragments collected after cultivating were afterwards frozen in liquid N2. Control thawed cells demonstrated viability.

Keywords: Tissue fragments, Cell migration, Passage, Viability, Tissue like structure

Characterization of heterotrophs from Icelandic waters

Hjorleifur Einarsson[1], Kristinn P. Magnusson[1,2], Arnheiður Eythorsdottir[1], Halldor G. Olafsson[3]

[1]University of Akureyri, IS 600, Akureyri, Iceland, Iceland, [2]The Icelandic Institute of Natural History, IS 600 Akureyri, Iceland, [3]BioPol ehf, IS 545 Skagastrond.

Corresponding author: Hjorleifur Einarsson, hei@unak.is

The main objective of this project was to isolate and culture protista of the Thraustochytriaceae family.

Sea-derived samples were collected and pure cultures of 39 strains of Thraustochytrids were isolated. The protists were cultivated at 25 °C for 4–7 days. The strains used glucose and glycerol as carbon sources. Total lipid content was approximately 10 % of biomass dry weight. Highest dry weight was obtained at 3.92 g/l. Fatty acid composition of DHA (C22:6-n3), DPA (C22:5-n6), EPA (20:5n-3) and ARA (20:4-n6) were highest at 37.5, 5.4, 6.2 and 19.7 % respectively. Concentration of carotenoids was highest 371 μg/g.
Thraustochytrium kinnei, and for the first time, *Sicyoidochytrium minutum* have been isolated and identified from Icelandic waters. These results encourage further studies of the strains and production on a bigger scale.

Keywords: Heterotrophs, Thraustochytrids, Isolation, Characterization, PUFA

Session: Posters

Primary cell cultures from *Sepia officinalis* embryos: preliminary results

Yann Bassaglia[1,2] and Sébastien Baratte[1,3]

[1] Muséum National d'Histoire Naturelle, DMPA, Lab. BOREA. UMR MNHN CNRS 7208-IRD 207-UPMC, [2] Univ. Paris Est Créteil-Val de Marne (UPEC), Paris, France, [3] Univ. Paris Sorbonne - Paris 4, Paris, France

Corresponding author: Yann Bassaglia, bassaglia@mnhn.fr

Sepia officinalis is an emerging model in evo-devo but cellular techniques are currently missing. We aim to develop and

characterize primary cultures from *S. officinalis* embryos as an experimental tool to better characterize the relationships between cell types during neuro-muscular development.
Different dissociation techniques and culture media derived from classical media (Leibovitz L-15, Medium 199) were tested. The best results were obtained after dissociation in 0.25 % collagenase and culture in a modified L-15 medium (~800 mOsm) on untreated plastic dishes. Cell adhesion was difficult but no significant amelioration was noted using gelatin-, laminin- or fibronectin-coated substrates.

In these conditions, cells from optic lobe (stage 25–30 after Lemaire) gave rise to neurite expansion, clearly suggesting their neuronal determination. Other cell types were observed in cell cultures from skin, mantle or arms; with the exception of pigmented cells derived from chromatophores or probable muscle cells, their cellular characterization remained largely uncertain.

This preliminary work is an encouraging starting point and should allow the establishment of reliable cultures from different *S. officinalis* cell types.

Keywords: Cephalopod, *Sepia officinalis*, Dissociated cells, Neuro-muscular differentiation

Anemonia viridis primary cell culture: a new tool for cnidarian studies

Stéphanie Barnay-Verdier, Diane Dall'osso, Nathalie Joli, Juliette Olivré, Fabrice Priouzeau, Thamilla Zamoum, Pierre-Laurent Merle, Paola Furla

UMR SAE 7138, UPMC/CNRS/MNHN/UNS, Equipe Symbiose Marine, Faculté des Sciences de Nice, Parc Valrose, 06108 Nice Cedex 02, France

Corresponding author: Stéphanie Barnay-Verdier, stephanie.barnay-verdier@upmc.fr

Member of the cnidarian phylum, the temperate symbiotic sea anemone *Anemonia viridis* is a relevant experimental model to investigate, in a post-genomics approach, the molecular and cellular events involved in the preservation or in the rupture of the symbiosis between the animal cells and their symbiotic microalgae, named zooxanthellae (Sabourault et al. 2009; Ganot et al. 2011; Moya et al. 2012).

In this aim, we developed a primary culture from *A. viridis* epidermal and gastrodermal cells. By adapting and optimizing previous published methods, i.e. spontaneous or chemical dissociations (Frank et al. 1994; Domart-Coulon et al. 2004), we extracted cells from whole tentacle or from a separated epithelial cell layers corresponding to the epiderm or the gastroderm. Each plating resulted in a heterogeneous primary culture of different cell types as discharged cnidocytes, free zooxanthella cells (*A. viridis* symbiotes) and many regular, small rounded and adherent cells (of 3–5 μm diameter). The different culture observations showed that this last cell group contains *A. viridis* epithelial undifferentiated cells. Moreover, PCR analyses conducted on primary cultures, maintained for 2 weeks, confirmed a specific signature of *A. viridis*. In parallel, we evaluated the cell viability of these cultures by vital staining. Serial dilutions, led during 4 weeks, of re-suspended small rounded cells isolated using chemical dissociation allowed us to obtain a homogenous primary culture of *A. viridis* epithelial undifferentiated cells.

The maintenance and the propagation of this homogenous primary cell culture for several weeks provide suitable model for in vitro cnidarian studies and preliminary step for further investigations on symbiosis mechanisms.

Keywords: Primary cell culture; Cnidarian; *Anemonia viridis*; Symbiosis

Primary cell culture of mantle of the black-lip pearl oyster *Pinctada margaritifera*

Nelly Schmitt, Marina Demoy-Schneider

Université de la Polynésie Française, Laboratoire de Biodiversité Terrestre et Marine (BIOTEM), EA 4239, Equipe Biologie Marine, BP 6570 98 717 Faa'a Aéroport, Tahiti

Corresponding author: marina.demoy-schneider@upf.pf

During the pearl-sac formation in the black-lip pearl oyster *Pinctada margaritifera*, it is well-known that cells are migrating from the grafted mantle to build the pearl-sac. It should be useful to observe in vitro, the interactions between the different mantle cell types and to study the effect of specific molecules on the mantle cells proliferation, in order to coat the nucleus beads with substances stimulating this process or to immerse the grafted mantle a solution containing such molecules. First, we need to obtain viable primary cell cultures from the mantle of the mollusk and this is the aim of the present study.

All the experiments were carried out with black-lip pearl oysters *Pincatada margaritifera* which were raised in Takapoto atoll, Tuamotu archipelago (French Polynesia). They were collected from culture longlines and then transported in a container (room temperature) thanks to a 1-h flight shipment to the laboratory (University of French Polynesia, Tahiti). They were then stored in filtered UV-sterilized seawater for 3 days. Small pieces (4 mm^2) were aseptically removed from the mantle of the oyster and then placed in small dishes in different culture media. The explants were cultured at 25 °C for several days, up to 16 days.

The primary cultures were observed daily under a light inverted microscope (×320 magnification). The results showed cells migrating from the explants 8 days after the beginning of the culture. Several different cell types were observed in the dishes: epithelial cells, hyalinocytes, fusiform "muscular-like" cells. The best results of confluent cells were obtained 11 days after the beginning of the culture then the cells progressively died.

Keywords: *Pinctada margaritifera*, Mantle, Cell Culture

Diversity of bacteria associated with the carnivorous sponge *Asbestopluma hypogea*

Samuel Dupont[1], Alyssa Carre-MLOUKA[1], Yan-yan Li[1], Jean Vacelet[2], Marie-Lise Bourguet-Kondracki[1]

[1] Laboratoire Molécules de Communication et Adaptation des Micro-organismes, UMR 7245 CNRS, Muséum National d'Histoire Naturelle, 57 rue Cuvier C.P. 54, 75005 Paris, France, [2] Centre d'Océanologie de Marseille, Aix-Marseille Université, CNRS UMR 6540 DIMAR, Station Marine d'Endoume, Rue de la Batterie des Lions, 13007 Marseille, France.

Corresponding author: Samuel Dupont, sdupont@mnhn.fr

Marine sponges of the genus *Asbestopluma* are carnivorous sponges originally found in deep sea waters. The first discovery of the species *Asbestopluma hypogea* in 1996 (Vacelet et al. 1996) in a littoral cave (20 m depth) off the Mediterranean coast, France, gave the great opportunity to study this rare species, which can be easily raised in an aquarium. Microscopic studies revealed that this tiny carnivorous sponge, which has no digestive tract, harbors numerous bacterial strains in its mesohyl. Aiming to obtain further insights into the role of these associated bacteria within the sponge host, we have investigated the microbial community associated with *A. hypogea* combining microscopic, cultural, metagenomic and chemical approaches.

Preliminary microscopic observations had revealed the presence of bacteria and archaea in *A. hypogea* tissues. Further studies (MET) showed the presence of bacteriocytes in the mesohyl of the sponge containing two morphotypes (rod shaped, coccoid). Culture-dependant and culture independent approaches were developed in order to estimate the cultivable and the total microbial flora of *A. hypogea*, respectively. Fifty-seven bacterial strains were isolated and identified using the 16S gene sequences. These sponge associated bacteria can be affiliated to three bacterial groups: *Proteobacteria*, *Firmicutes*, and *Flavobacteriacea*. Fluorescence in situ hybridization studies are in progress to allow the localization of bacteria isolates in the sponges cells.Evaluation of the role of these bacteria in nutrition or as modulators of microbial associations was investigated through antioxidant, antimicrobial and chitinase assays. Bacterial strains *Paracoccus sp.* S2, *Streptomyces sp*. ASB1 and *Joostella sp*. ASB7 are of particular interest since they revealed significant activities in the three assays.

Further chemical investigations are underway to identify the molecule(s) responsible for these activities and to determine their contribution to the associations.

Keywords: *Asbestopluma hypogea,* Carnivorous Sponge, Bacteria, microscopy

Cytotoxic effects of in vitro exposure to triclosan on the marine gastropod *Haliotis tuberculata*

Béatrice Gaume[1,2], Nathalie Bourgougnon[1], Stéphanie Auzoux-Bordenave[2,3], Benoit Roig[4], Barbara Le Bot[4], Gilles Bedoux[1]

[1] Laboratoire de Biotechnologie et Chimie Marines, EA3884, Université de Bretagne-Sud (Université Européenne de Bretagne), IUEM, Vannes, France, [2] UMR BOREA (Biologie des Organismes et Ecosystèmes Aquatiques), MNHN/CNRS 7208/IRD 207/UPMC, Muséum national d'Histoire naturelle, Station de Biologie Marine de Concarneau, Concarneau, France, [3] Université Pierre et Marie Curie Paris, 4 place Jussieu, 75 005 Paris, France, [4] U1085 IRSET/LERES EHESP, Advanced School of Public Health, Rennes, France

Corresponding author: Gilles Bedoux, gilles.bedoux@univ-ubs.fr

Human activity has been increasing exponentially the number and concentrations of molecules rejected in the environment. Some of these chemicals compounds are totally degraded in sewage treatment plants; others are partially eliminated and then found in rivers and ocean. Aquatic flora and fauna are the first organisms impacted by this anthropogenic pollution that has effects on the nervous and endocrine systems. Triclosan (2,4,4′-trichloro-2′-hydroxy-diphenyl ether; TCS) is an antibacterial agent incorporated in a wide variety of household and personal care products. Because of its partial elimination in sewage treatment plants, it is one of the most commonly detected organic wastewater compounds for frequency and concentration. Moreover, due to its high hydrophobicity, TCS accumulates in fatty tissues of aquatic organisms. Under these conditions, methyl-triclosan (2,4,4′-trichloro-2′-methoxydiphenyl ether, MTCS) is the main transformation product of TCS. Although MTCS is generally less prevalent in the environment than TCS, its mechanism of action is similar and can occur at measurable levels.

Very few studies are available about TCS toxicity on marine mollusks. The European abalone *Haliotis tuberculata* is a marine gastropod of economic interest which inhabits rocky shores in Brittany. Since abalone is particularly exposed to anthropogenic pollution, it is a relevant model for ecotoxicological studies. The aim of our study was to assess the acute cytotoxicity of TCS in short-term in vitro experiments. Primary cultures of hemocytes—circulating cells of abalone—and gill cells were incubated with TCS concentrations ranging from 0 to 10 μM for 24–48 h. The cytotoxicity of TCS was evaluated through the cell viability using the XTT assay.

The results showed that morphology and density of hemocytes are affected from a concentration of 8 μM TCS. Using the XTT reduction assay, TCS has been demonstrated to decrease hemocyte metabolism activity in a dose- and time-dependent exposure. The IC_{50} was evaluated at 6 μM for both hemocyte and gill cells after a 24 h-incubation with TCS. A significant cytotoxicity of MTCS was also observed from 4 μM in 24 h-old hemocyte culture.

Our results reveal a toxic effect of TCS on both immune (hemocytes) and respiratory cells (gill cells) of the abalone, a marine species exposed to anthropogenic pollution.

Keywords: Triclosan, Methyl-triclosan, Antibacterial, Cytotoxicity, Cell culture, Marine gastropod, *Haliotis tuberculata*

Early assessment of the quality of cryopreserved *Pinctada margaritifera* spermatozoa

Neïla Guelaï[1], Nelly Schmitt[1], Gaël Le Pennec[2], Marina Demoy-Schneider[1]

[1]Université de la Polynésie Française, Laboratoire de Biodiversité Terrestre et Marine (BIOTEM), EA 4239, Equipe Biologie Marine, BP 6570 98 717 Faa'a Aéroport, Tahiti, Polynésie française, [2] Université Bretagne-Sud : Laboratoire de Biotechnologies et Chimie Marines- Rue Saint Maudé 56 321 Lorient cedex, France

Corresponding author: Marina Demoy-Schneider, marina.demoy-schneider@upf.pf

Spermatozoa cryopreservation is a useful tool for genetic improvement and has been applied to several bivalve mollusc species. This technology would allow preserving the gametes of individuals selected for their high growth capacity or the quality of their pearl and thus provide significant benefits to the

cultured black pearl industry. Sperm freezing requires the control of different steps: preparation of breeders, sperm collection, evaluation of sperm quality and the freezing process itself.

The objective of this study is to estimate the quality of cryopreserved spermatozoa immediately after thawing. Therefore, different criteria need to be evaluated such as the ultrastructure, concentration, movement characteristics of the sperm before and after cryopreservation. Sperm was manually collected after natural "shedding" from the gonopore. After appropriate dilution in swimming media, spermatozoa movement characteristics were estimated under light microscopy using CASA image analysis. Ultrathin sections were prepared for TEM examination. The presence of parvalbumin-like protein (indicator of spermatozoa maturity) was immunodetected after electrophoresis.

Concerning the freezing process, sperm was diluted in a cryoprotectant then drawn into semen straws. After equilibration at room temperature then in liquid nitrogen steam, the straws were immersed in the liquid nitrogen at least for 2 h and finally thawed at room temperature.

Our results showed that, spermatozoa can be reactivated in alkaline media and are able to restore motility after cryopreservation.

Keywords: Black-lip pearl oyster, *Pinctada margaritifera*, Cryopreservation, sperm quality

Haemocyte primary-culture from three mollusc species and its application in ecotoxicology

Charles Le Pabic*, Antoine Mottier*, Katherine Costil, Noussithé Koueta, Jean-Marc Lebel, Antoine Serpentini

CNRS-INEE FRE 3484 BioMEA (Biologie de Mollusques Marins et des Ecosystèmes Associés), University of Caen Basse-Normandie, Esplanade de la Paix, 14032 Caen Cedex, France. * These authors contributed equally to the work.

Corresponding author: Charles Le Pabic, charles.lepabic@unicaen.fr

Haemocytes play a fundamental role in invertebrate immune system and are responsible for different types of immune responses such as phagocytosis, pathogen hydrolysis or phenoloxydase cascade. These cells could potentially be affected by contaminants such as pesticides and metals. To better understand impacts of contaminants on mollusc immune system, in vitro primary-culture of haemocytes could be a useful tool (Mottin et al. 2010; Latire et al. 2012). The aims of this work were to improve culture parameters for haemocytes from three species of marine molluscs (*Crassostrea gigas*, *Sepia officinalis* and *Haliotis tuberculata*) in order to use those cells for ecotoxicity assays.

To assess our cellular culture conditions, MTT reduction assay or Water Soluble Tetrazolium salts (WST-1) assays were performed after different times of culture. After the validation of culture parameters, viability assays and other biomarkers were conducted to assess the effects of different types of contaminants.

Experiments on *Crassostrea gigas* haemocytes showed difficulty to maintain these cells in culture. Different cell concentrations and different kinds of culture media were tested but a high decrease of cell viability was observed on the first 48 h of culture. In *Sepia officinalis,* haemocyte primary-culture is poorly documented and different parameters were thus tested. Combination of modified L-15, Hank's 199 and DMEM media, and two temperatures were assessed. Results showed that the combination of L-15 medium and the temperature of 15 °C was the best one for cuttlefish haemocyte primary-culture. First experiments carried out on cuttlefish haemocytes under zinc contamination showed a negative effect on lysosomal system after 48 h of exposure.

Parameters for *Haliotis tuberculata* haemocyte primary-culture are well known and those cells could be maintained for 10 days in modified Hank's 199 medium without any decrease of viability. Effects of an herbicide (glyphosate) and a metal (zinc) were tested after 10 days of exposures. Glyphosate appeared to have no effect on haemocyte viability even at very high doses (e.g. 100,000 μg L^{-1}) whereas an EC_{50} of 6,300 μg L^{-1} could be calculated for zinc.

This approach will allow us to compare haemocyte responses from three species of molluscs which differ phylogenetically and ecologically.

Keywords: Haemocytes, primary culture, Molluscs, ecotoxicology

Development of primary cell cultures from sea urchin gonads

Silvia Mercurio, Cristiano Di Benedetto, Michela Sugni, M. Daniela Candia Carnevali

University of Milan, Department of Life Sciences, Via Celoria, 26 - 20133 Milano, Italy.

Corresponding author: Silvia Mercurio: silvia.mercurio@unimi.it

The Mediterranean sea urchin *Paracentrotus lividus* is one of the favorite and most used experimental model in developmental biology. Taking into account the possible advantages and applied implications of in vitro studies (Rinkevich 1999), first attempts to develop primary cell cultures from gonads of this species were carried out. Gonads were collected, dissected into small pieces and incubated in sterile Ca^{2+} Mg^{2+} Free Sea Water with 0.5 mg/mL collagenase for 1 h. The resulting cell suspensions were filtered through 50 μm nylon gauze, then centrifuged (330 g × 6 min) and the cell pellets were resuspended in culture medium. The obtained cell phenotypes (germ cells and nutritive phagocytes) were determined by detailed histological analysis. Three different modified culture media were tested: Leibovitz-15 (L-15), Medium 199 (M199) and Minimum Essential Medium Eagle (MEM). According to cell morphology and viability tests (direct cell counting using "Burker chamber" coupled with Trypan blue exclusion test), L-15 appeared to be the most suitable medium for cell growth and survival. Particularly, cell viability resulted significantly higher in L-15 cell cultures (One-way ANOVA: $P < 0.001$) at all considered time points (2 days, 1 and 2 weeks). M199 and MEM media were not effective probably because they revealed pH instability, as also reported by Mulford and Austin (1998). Various substrates were tested. Gonad cells adhered only on poly-L-lysine substrate, whereas we did not find any

improvements in terms of cell adhesion using mammalian collagen, gelatin and sea urchin insoluble collagen substrates. To stimulate cell growth and survival L-15 medium was supplemented with: (1) inactivated standard Fetal Calf Serum (FCS) or (2) a "sea urchin Pluteus Estract" (PE) specifically developed in our lab. In FCS-supplemented cell cultures signs of degeneration on were observed early and cell viability was significantly lower than in unsupplemented cultures (One-way ANOVA: $P < 0.05$), suggesting that for sea urchin gonad cells this extract does not improve cell conditions, as, on the contrary, was reported for other marine invertebrates (Mulford and Austin 1998; Walton and Smith 1999; Odintsova et al. 2005). Different concentrations (up to 50 μg/mL) of PE were tested but no improvement in cell conditions was obtained as well. PE apparently increased and accelerated processes of cell death even at low concentration. This preliminary study suggests that it is possible to develop primary cell cultures from sea urchin gonads and maintain these cells under in vitro conditions for more than 1 month. Overall, our findings represent an important starting point for the establishment of proliferative primary cell culture from *P. lividus* gonads.

Keywords: Sea urchin, Gonads, Primary cell culture, Medium, Viability

References

Mulford AL, Austin B (1998). Development of primary cell cultures from *Nephrops norvegicus*. Methods in Cell Science, 19: 269–275

Odintsova NA, Dolmatov I Yu., Mashanov VS (2005). Regenerating holothurian tissues as a source of cells for long-term cultures. Marine Biology, 146: 915–921

Rinkevich B (1999). Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. Journal of Biotechnology, 70: 133–153

Walton A, Smith VJ (1999). Primary cell culture of the hyaline haemocytes from marine decapods. Fish and Shellfish Immunology, 9: 181–194

Characterization of abalone *Haliotis tuberculata–Vibrio harveyi* interactions in gill primary cultures

Delphine Pichon[1], Benoit Cudennec[2], Sylvain Huchette[3], Christine Paillard[4], Stéphanie Auzoux-Bordenave[1,5]

[1]Station de Biologie Marine, Muséum National d'Histoire Naturelle, DMPA, UMR BOREA 7208 CNRS/MNHN/IRD/UPMC, 29900 Concarneau;[2] Laboratoire ProBioGEM, Université Lille1, Villeneuve d'Asq, France, [3] France-Haliotis 29880 Plouguerneau, [4] LEMAR Université de Bretagne occidentale, Brest, France, [5] Université Pierre et Marie Curie Paris VI, 4 place Jussieu, 75005 Paris, France

Corresponding author: Delphine Pichon, pichon@mnhn.fr

The decline of European abalone *Haliotis tuberculata* populations have been associated with various parasites among them the bacteria of the genus *Vibrio*. Following the summer mortalities of 1998 and 2000 in France, *Vibrio harveyi* strains were isolated from both farmed and wild abalones, allowing in vivo and in vitro studies on the interactions between abalone *H. tuberculata* and *Vibrio harveyi*.

This work reports the development of primary cell culture from abalone gill tissue, a target tissue for bacterial infection, and their use for in vitro study of host cell—*Vibrio harveyi* interactions. Gill cells originated from 4-day-old explant primary cultures were successfully sub-cultured in multi-well plates and maintained in vitro for up to 24 days. Cytological parameters, cell morphology and viability were monitored over time using flow cytometry analysis and semi-quantitative assay (XTT). Then, gill cell cultures were used to investigate in vitro the mode of action of *V. harveyi*. The effects of two bacterial strains were evaluated on gill cells: a pathogen bacterial strain ORM4 which is responsible of abalone mortalities and LMG7890 a non-pathogenic strain. Cellular responses of gill cells exposed to increasing concentrations of bacteria were evaluated by measuring mitochondrial activity (XTT assay) and phenoloxydase activity, an enzyme which is strongly involved in immune response. The abiltity of gill cells to phagocyte *V. harveyi* was studied by flow cytometry and gill cells-*Vibrio harveyi* interactions were characterized using fluorescent microscopy and transmission electron microscopy.

During phagocytosis process we evidenced that *Vibrio harveyi* bacteria induced significant changes in gill cells metabolism and immune response. Together, the results showed that primary cell cultures from abalone gills are suitable for in vitro study of host-pathogen interactions, providing complementary assays to in vivo experiments.

Keywords: *Haliotis tuberculata*, *Vibrio harveyi*, Gills cell culture, Pathogenicity

Establishment of a primary cell culture from *Crangon crangon* and their characteristics

Frauke Symanowski, Bianka Grunow

Fraunhofer Research Institution for Marine Biotechnology, Paul-Ehrlich-Straße 1-3, 23562 Lübeck, Germany

Corresponding author:
frauke.symanowski@emb.fraunhofer.de

The present study reports a culture technique for hepatopancreatal tissues of brown shrimp (*Crangon crangon*). This commercially important species of shrimp is widely distributed along the European coast from the White Sea to Morocco within the Atlantic and North Sea as well as throughout the Mediterranean and Black Sea. Hepatopancreatal tissues were isolated and digested by a trypsin–EDTA treatment and a mechanical dissection with scissor, resulting in a slowly but continuously proliferating cell culture. The cells were stable in the process of long-term cultivation over 6 months. Light and electron microscopically studies as well as red oil staining were carried out in order to characterize the cells and the isolated tissue of the brown shrimp. Two types of cells were recognized: dark pigmented cells with highly different shape and colorless spherule cells with vacuoles containing lipid droplets.

These results contribute to the development of invertebrate cell culture and provide an important tool for research of *Crangon crangon* populations regarding to virus infection and other immunological studies.

Keywords: Cell culture, Crangon, Shrimp, Marine invertebrates