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REPORT OF MEETING

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Studying stem cells and differentiation with ACME dissociation

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How do stem cells give rise to a myriad of different cell types? The study of stem cells and their differentiation processes is being largely enabled by single-cell transcriptomic approaches. Planarians are an ideal model to answer this question, since they have pluripotent stem cells that give rise to all adult differentiated cell types constantly. Thus, an adult planarian contains a snapshot of the entire process, with stem cells, all differentiated cells and every intermediate differentiating cell in between.

To answer this question, we have developed ACME (ACetic-MEthanol) dissociation. Single-cell sequencing technologies are limited by the need to dissociate fresh samples that can only be fixed at later stages. This renders profiling cell types of non-model organisms extremely challenging. Our novel method, ACME, is a cell dissociation approach that fixes cells as they are being dissociated. ACME-dissociated cells have high RNA integrity, can be cryopreserved multiple times, can be sorted by Fluorescence-Activated Cell Sorting (FACS) and are permeable, enabling combinatorial single-cell transcriptomic approaches. ACME is based on affordable reagents, can be done in most laboratories and even in the field, and thus will accelerate our knowledge of stem cells and differentiated cell types across the tree of life.

A *Hydra* Claudin cell-cell contact protein is involved in epithelial tissue dynamics, regeneration, and osmoregulation

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Claudins are major components of tight junctions in vertebrates, and they are involved in apical junctional complexes in some invertebrate bilaterians. It is unclear, when the function of Claudins in cell adhesion and junction formation evolved in basal, pre-bilaterian metazoans, and to which degree stem cell based processes such as dynamic tissue replacement, morphogenesis, and regeneration require Claudin proteins in those ancestral taxa. We identified 38 potential orthologues of the claudin and claudin-like gene familes in the cnidarian polyp model *Hydra*. Their single-cell transcriptome expression patterns suggest specific assembly patterns in apical septate junctions built between the various cell types. In order to approach the potential functions of Claudin proteins in adult epithelial stem cells in *Hydra*, we went on to carry out a detailed analysis of gene expression, protein localization, and functional interference of one selected claudin gene, *Hydra* claudin1. In situ hybridization experiments showed that claudin1 is expressed uniformly at a moderate level throughout the ectodermal epithelial layer. It is, however, up-regulated in the tip of the hypostome, at the basis of tentacles, and just distal to the basal disc. During bud formation and head regeneration, up-regulation of

claudin1 expression is associated with the formation of differentiated tentacle, hypostome and foot tissues. Transgenic *Hydra* expressing a Claudin1-GFP fusion protein clearly demonstrate specific protein localization in apical septate junctions throughout the entire polyp body. Using an optimized siRNA protocol, we were able to efficiently knock-down claudin1. These animals show disruptions of ectodermal epithelial organization and a disturbed ultra-structure in their septate junctions. They also exhibited strongly reduced capacities for head and foot regeneration. Furthermore, preliminary data suggest that claudin1 knock-down reduces the ability of the ectodermal epithelial layer to maintain normal osmoregulation. In conclusion, our study is the first detailed functional characterization of an ancestral Claudin cell-cell-contact protein acting in epithelial stem cells in maintaining tissue integrity and in dynamic morphogenetic processes.

Evolutionary dynamics of the *myc* gene family and functional conservation of an ancestral and structurally unique *Hydra* Myc protein

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Among stemness genes, the proto-oncogene myc has been intensively studied over the last decades, investigated primarily in vertebrate and cell culture systems. Myc transcription factors are known to control fundamental cellular processes such as cell proliferation, cell cycle and stem cell maintenance. In addition, Myc requires hetero-dimerization with the protein Max to be functional, and both co-regulate thousands of target genes. However, the ancestral origins of Myc and Max proteins and their evolutionary dynamics throughout all metazoans remain poorly understood. In the present study, Myc and Max conservation across the Metazoan tree will be shown. Vertebrates possess a diversification of Myc proteins commonly known as c-Myc, l-Myc and n-Myc. In comparison, the freshwater polyp *Hydra* encodes four distinct myc genes in its genome. Structural and biochemical characterization unraveled HyMyc1 and HyMyc2 to share high similarities with c-Myc of vertebrates, also in terms of acting in adult stem cell maintenance. An additional Myc protein in *Hydra*, HyMyc3, however seems to be highly divergent, totally lacking the common N-terminal domain containing four conserved Myc-boxes. Single-cell transcriptome sequencing clearly showed that HyMyc3 is active in a distinct population of interstitial precursor cells committed for nerve and gland cell differentiation. We currently speculate that it may act in these cells as a dominant-negative factor counteracting the stemness action of HyMyc1 and HyMyc2, and thereby allowing the implementation of a differentiation program. Furthermore, in order to define whether principal functions of the oncogenic vertebrate Myc are associated with this ancestral HyMyc3 protein, we performed cell transformation assay in avian fibroblast cultures. Here, HyMyc3 showed an unexpected high potential for oncogenic transformation. These oncogenic properties and aspects of transcriptional activation will be discussed in relation to 3D structure modeling of HyMyc3.

Keywords: Stem cell; Myc; oncogene; *Hydra*, evolution

Marine collagens on the development of biomaterials for tridimensional culture of stem cells towards tissue regeneration

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Tissue engineering is established in the last decades as a strategy aiming the regeneration of tissues and organs, to change the paradigm to regenerative medicine. This strategy relies on the design of a temporary artificial extracellular matrix where cells are cultured in a tridimensional way, receiving appropriate cues to produce new tissues. Many materials have been explored to produce those matrices – named scaffolds – and in recent years marine origin compounds are arising as a valuable, safe and sustainable alternative. In particular, marine origin collagens have been used on the development of tissue engineering scaffolds by freeze-drying, 3D printing and chemical crosslinking, among other techniques. Fish collagens are widely studied, being produced from fish by-products as valorization approach, but marine invertebrates have been also explored, namely marine sponges and jellyfish. This talk discusses examples of scaffolds comprising fish collagens, shown to support the differentiation of human stem cells towards osteogenic and chondrogenic lineages. Moreover, other biomaterials produced with collagens from marine sponges and jellyfish, combines with polysaccharides are also addressed, capable to support the culture and proliferation of different cell lines. One can hypothesize that these polymeric and composite structures can also offer templates for the culture of other cells, namely marine invertebrate stem cells, to support advanced marine biotechnology studies.

Sub-lethal 5-Fluorouracil treatment promotes transcriptional profile changes in the piwiA-/soxp1-positive planarian stem cells

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Planarians possess extraordinary biological performance, such as the possibility to regenerate any missing body part, thanks to the presence of an abundant population of stem cells, the so called neoblasts. Although neoblasts share similar morphology, several findings indicate a complex transcriptionally heterogeneous population of cells. Contrasting models picture them as a series of populations with a progressive fate restriction or as a single type of cells that encompass different transcriptional states through the cells cycle. Challenging conditions such as sub-lethal X-ray treatment, where the depletion of stem cells is rescued by a repopulation process, are ideal model

systems to deepen the understanding of the mechanisms orchestrating neoblast specialization and potency. Here, we identified a further challenging condition by exposing planarians to different concentrations of 5-fluorouracil (5-FU), a genotoxic drug. We identified a sub-lethal concentration of 5-FU that induces depletion of stem cells and S-phase slowdown that triggers transcriptional changes in the piwiA/soxp-1-positive neoblasts. At later time, some piwiA-/sox-P1-positive neoblasts reappear at the ventral side of the animals, close to the nerve cords, and repopulate the body reconstituting the complex neoblast system. Our data support the possibility that some neoblasts, in the earlier steps of commitment (or even post mitotic early progeny), could modulate their expression profile, so reacquiring a broader differentiative potential.

Analysis of cell hierarchies in the regenerative flatworm *Macrostomum lignano* using single-cell transcriptomics and stem cell-specific transgenic lines

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Marine flatworm *Macrostomum lignano* is an excellent model organism to study the mechanisms of stem cell regulation and regeneration. This animal can regenerate most of its body parts, it is easy to culture in laboratory conditions, and it is possible to create transgenic *M. lignano* lines. We use this model to understand how stem cells (neoblasts) in this animal make all other cell types and regenerate tissues and organs. Towards this, we have generated several transgenic *M. lignano* lines labeling specifically neoblast by CRIPS/Cas9-mediated knock-in of various fluorescent protein-encoding genes in frame with a histone 2A variant gene that is expressed specifically in neoblasts. Single cell transcriptomic analysis of FACS-isolated neoblasts, as well as all cells of the animal allowed us to identify major cell types in *M. lignano* and establish relations between neoblasts and differentiated cells. These data prove instructive for generating experimental hypotheses for further in-depth investigation of stem cell regulation in *M. lignano*.

Absence of larval regeneration in the highly regenerative crinoid *Antedon mediterranea*

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Regeneration is a fundamental mechanism among metazoan, being present in most existing phyla. Echinoderms are marine deuterostomes, therefore phylogenetically close to Vertebrates, and they are characterized by extraordinary regenerative abilities, both in adults and larval stages. Larval regeneration (and larval cloning) is well documented for all echinoderm classes, except Crinoidea, the most basal taxon. Therefore, the aim of this work was to assess if the larval stage (doliolaria) of the crinoid *Antedon mediterranea*, whose adults are perfectly able to regenerate almost any tissue, can regenerate as well. In normal conditions, free-swimming *A. mediterranea* doliolaria attaches to the substratum and develops in a temporary post-metamorphic stalked stage (pentacrinoid) provided with an apical calix; this latter will eventually detach originating the free-swimming adult individual. Adult specimens of *A. mediterranea* were collected at Le Grazie (La Spezia Gulf, SP, Liguria). After hatching, doliolaria larvae were collected and transversally bisected with surgical blades, thus obtaining anterior and posterior halves. The fragments were monitored for 2-3 weeks and the survival rate was registered and compared to non-bisected doliolariae. We defined different "developmental" stages (seven for the anterior fragments and five for the posterior ones) through which both fragments go during the days post-amputation. Each stage was characterized by stereomicroscopy, Scanning Electron Microscopy (SEM), light microscopy and Transmission Electron Microscopy (TEM). Results indicate that less than 50% of the bisected larvae survived after 3 weeks and none of the surviving halves was able to completely regenerate. Rather, after a wound-healing phase each half continued its pre-determined development and the obtained post-metamorphic stage lacked structures deriving from the missing half: anterior fragments originated a stalk without a calix whereas the posterior halves produced a calix without a stalk. In terms of inner microscopic anatomy, each of the fragment properly developed the specific tissues normally present in the corresponding half of the larva (i.e. the stalk produced by the anterior part developed serial columnal ossicles and a well-defined longitudinal nerve). Light microscopy and Transmission Electron Microscopy were helpful tools to highlight the different cellular types involved in this development. These data suggest that: doliolaria cells are strictly committed to their original fate; cellular plasticity/dedifferentiation is temporary blocked and/or "stem cells" are missing or in a "stand-by" state. Whatever the mechanism, this is "suddenly" and remarkably reverted just upon metamorphosis, as freshly metamorphosed individuals are already able to regenerate their tissues/structures. Considering the basal phylogenetic position of Crinoidea these results are particularly significant to better understand the evolutionary trajectories which led to gain or loss of (larval) regenerative abilities. More studies could also shed light on the correlation between regeneration in adults and regeneration in early stages.

Sweet tunicate blood cells: a glycan profiling of hemocytes in three ascidian species

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Ascidians are invertebrate chordates and may reveal evolutionary origins of vertebrate traits

including cellular immunity, tissue rejection and self-renewal, all functions executed by ascidian blood cells. Understanding their individual properties, lineage homologies and functional plasticity is, however, limited by a lack of cytochemical and molecular markers. We performed a lectin-based glycan profiling of hemocytes in three ascidian species to distinguish different blood cell populations and mirror their relatedness. We found differing repertoires of species specific glycans for blood cells thought homologous in their function. Within species, characteristic glycans or glycan combinations mark hemocyte types and support their hematopoietic relatedness or distinguish maturation stages. Strikingly, *Ciona* and *Phallusia* hemoblasts have few carbohydrate decorations and drastically differ from differentiated cells, likewise phagocytes from cytotoxic cells as compared to *Botryllus*, where a complex role of hemocytes in asexual self-renewal and allorecognition may involve carbohydrates. Cytotoxic cells generally carry most decorations. Within cell types, specific carbohydrates reside on the cell surface including amoeboid extentions while others are within granules possibly marking molecules important in cytotoxicity and crosslinking. Taken together, these carbohydrate biosensors should further the molecular and functional characterization of the outstanding properties of the different hemocytes in genetically accessible ascidian species.

Keywords: Tunicates; ascidians; hemocytes; blood cells; lectin staining; glycans; carbohydrates; *Ciona*; *Phallusia*; *Botryllus*

Stem cells in the colonial ascidian *Botryllus schlosseri*: contribution to asexual development and morphological characterization of their niches

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Colonial ascidians are the only chordates able to regenerate whole organisms from a small pool of circulating stem cells. Among them, *Botryllus schlosseri* can regenerate the entire colony, once all the zooids are removed, starting from circulating stem cells (SCs). Moreover, it constitutively develops individuals (zooids) by budding, from a small number of multipotent cells located in the body wall, throughout its entire life. It is still unclear whether the initial bud rudiment has the capability to form all the tissues of newly developing zooid, whether SC participate to organogenesis, and which molecular machinery governs pluripotency in this chordate. Moreover, stem cell niches have been identified in this species, but their precise morphological characterization is still missing.

We characterized SC niches by means of histology, 3D reconstructions and observation of whole mount-fixed specimens, verifying that candidate SCs undergo mitosis and differentiation in the niches. We followed the contribution of candidate SCs to bud organogenesis in-vivo, founding that candidate SCs can proliferate and localize in different territories and epithelia, undergoing mesenchymal-epithelial transition. Candidate SCs sorted by FACS and injected into compatible colonies confirmed these results. In conclusion, we carefully described stem cell niches and evidenced where and how candidate SCs contribute to bud organogenesis.

Isolation of immune cells and hematopoietic stem cells from the tunicate model *Botryllus schlosseri*

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The mechanisms that sustain immunological non-reactivity are the basis for understanding the maintenance of tissue in syngeneic and allogeneic settings. While most transplantation rejection occurs due to the adaptive immune response, the pro-inflammatory response of innate immunity is necessary for the activation of adaptive immunity - both in syngeneic and allogeneic settings. Here we studied the hematopoietic and immune systems of *Botryllus schlosseri*, a colonial tunicate that has vasculature, circulating blood cells, and interesting characteristics of stem cell biology and immunity. Self-recognition between genetically compatible B. schlosseri colonies leads to the formation of natural parabionts with shared circulation, whereas incompatible colonies reject each other. By means of flow-cytometry in combination with screened antibodies by Cytof, lectins, and fluorescent enzymatic reagents, we isolated 34 B. schlosseri cell populations. Using whole-transcriptome sequencing of defined cell populations, and diverse functional assays, we identified Hematopoietic Stem Cells (HSC), progenitors, immune-effector cells, and the HSC-niche.

Our study implies that the HSC and myeloid lineages emerged in a common ancestor of tunicates and vertebrates and suggests that hematopoietic bone marrow and the *B. schlosseri* endostyle niche evolved from the same origin (1-2). These findings taken together, make the *B. schlosseri* as a full model for HSCs transplantation and immune system modulation model.

Interestingly, since the methods developed in the project for cell isolation and immune functional assays are species non-specific, we could translate this research to a variety of non-classical model organisms. This including fish, urchins, anemones and even corals (3-6).

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Preliminary data on senescence in haemocytes of the colonial ascidian *Botryllus schlosseri*

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Senescence is a cellular response to damage that limits the proliferation of aged or effete cells and plays physiological roles as it is required for tissue homeostasis.

Colonies of the protochordate *Botryllus schlosseri*, undergo cyclical generation changes or takeovers (TOs) during which adult zooids are replaced by their buds reaching adulthood. The period of time between two TOs is referred to as blastogenetic cycle. During the TO, cells of adult zooid tissues die by apoptosis and are cleared by circulating phagocytes that, in turn, undergo phagocytosis-induced apoptosis and are cleared by new phagocytes in a recurrent, apparently endless, process. In the present work, we demonstrate that phagocytes, after the engulfment of effete phagocytes enter a senescence status and home, in the following mid-cycle, in the ventral islands, on both sides of the endostyle, where undifferentiated (stem) cells are also found. From these sites, senescent cells cross the peribranchial epithelium and are released in the peribranchial cavity where they will be expelled with the exhalant water.

Studies on Turkish coastlines' botryllid ascidians

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Tunicates are hermaphrodite, filter feeder invertebrate organisms that have an essential role in the coastal ecosystem and distributed worldwide oceans from tropic to polar regions and continue to spread via anthropogenic actions. They have both sexual and asexual reproduction and can undergo whole-body regeneration; those features make them perfect model organisms for stem cell-related studies such as regeneration, aging, development, allorecognition. Despite their unique features, botryllid ascidians are understudied. So far, 55 botryllid ascidians species have been identified. Recent studies showed that only morphological identification is not enough to discriminate the species since most botryllid ascidian species show similar morphologies. Also, it is shown that the most recent mtDNA (COI) analyses give preliminary results; however, since evolution is a gradual and continuous process, there should be more comprehensive studies for the species identification of the botryllid group. Different methods should be combined to understand their biology better and benefit from them as model organisms.

In IMS-METU, we have been doing regular sampling and monitoring along the Mediterranean coastline of Turkey for the last five years. We simultaneously observe botryllid ascidian species diversity, distribution, and fluctuations over the year with environmental changes, along with barcoding efforts. We also culture them in our aquaculture conditions and detail the comparative blastogenic cycle and whole-body regeneration, monitoring each morphology of the same or different species. Besides, we also collect ecologic, biologic, and genetic data of most botryllid species on a regional basis. Among all the species that have been monitored in the region, Botrylloides anceps was the ascidian species that grew nearly two years in the culture condition and the best model for our regeneration studies. For the future periods, it is planned to make sampling for botryllid ascidians from all coastline of Turkey, including the Mediterranean Sea, the Aegean Sea, the Marmara Sea, and the Black Sea. In this context, the determination of botryllid ascidians by using classical taxonomic and DNA barcoding methods aims to identify possible new species. In addition, it is aimed to determine the species that can be used as an indicator of water quality or pollution and investigate the potential effects of environmental factors on species diversity distribution. In the range of these studies, botryllid ascidians will be examined from an ecological and genetic perspective. Also, this process aims to designate the distribution of *Botryllus schlosseri*, which is of Mediterranean origin and distributed worldwide, on the Turkish coasts. Despite the extensive documentation of the presence in all Turkish seas, detailed genetic profiles of the species have not been studied. For these reasons, detecting single nucleotide polymorphism of populations using next-generation sequencing methods and interpretation of species' adaptive features according to detected variants and investigating its evolutionary history is valuable on both local and global scales.