Surfing with the tunicates into the post-genome era

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This year is the centenary of Edward G. Conklin's signal findings in embryology: the elucidation of complete cell lineages and the discovery of localized maternal determinants. Conklin used ascidian embryos to elucidate universal principles in embryology. A century later, ascidians, or sea squirts, have not only entered the post-genome era, but in many ways are leading the way to the promise of a "systems-level" understanding of complex processes such as notochord formation, neurogenesis, and even behavior.

In July 2005, the international tunicate research community was convened by Bill Smith (University of California, Santa Barbara) and Billie Swalla (University of Washington) at the University of California at Santa Barbara to discuss recent advances in the field. During the course of the meeting, it became increasingly clear that tunicates are emerging from the shadows of the more popular model organisms to become serious contenders in the illumination of the basic principles underlying morphogenesis.

"Tunicate" is the common name for the subphylum Urochordata, which includes ascidians, appendicularians (larvaceans), sorberaceans, and salps, and they are a sister group of vertebrates, including humans. They possess a notochord and dorsal neural tube in the tadpole-type larva or adult, and gill slits in the adult, features characteristic of all chordates. Since Conklin's work, ascidians have provided a classic experimental system to study cellular and molecular mechanisms underlying developmental fate determination and morphogenesis (Satoh et al. 2003). The emergence of ascidians into the modern era was precipitated by the assembly of the draft genome of *Ciona intestinalis* (Dehal et al. 2002). Many of the talks at the meeting were devoted to providing updates on the remarkable progress in making the *Ciona* genome one of the very best annotated of all animal genomes.

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Genomics

The *C. intestinalis* genome is composed of ~160 Mb and contains ~16,000 protein-coding genes that represent the basic set of chordate genes without the extensive gene duplications seen in vertebrates (Dehal et al. 2002). Most of the *C. intestinalis* gene models are supported by identified cDNAs. Several advances in genome annotation were announced. First, the Department of Energy Joint Genome Institute released a new and improved assembly (version 2) of the *C. intestinalis* genome (http://shake.jgi-psf.org/ciona2). Second, Kerrin Small (Stanford University) presented the genome sequence assembly of a second *Ciona* species, *Ciona savignyi* (http://www.broad.mit.edu/annotation/ciona). Third, Jan Vogel and Abel Ureta-Vidal (Wellcome Trust Genome Campus) reported a new *C. intestinalis* genome annotation that is available on the popular ENSEMBL Web site (http://www.ensembl.org). Fourth, Shigeki Fujiwara (Kochi University) reported the use of comprehensive microarray assays to obtain extensive temporal patterns of gene activity. Similarly, Lixy Yamada (Kyoto University) reported the use of these microarrays for determining the spatial patterns of gene expression within individual blastomeres in sequentially staged embryos. Finally, Eiichi Shoguchi (Kyoto University) reported the use of FISH analysis to map 169 BAC clones onto the 14 pairs of chromosomes in *C. intestinalis*.

The present collation of the sequence assembly and physical map covers more than half of the entire *Ciona* genome. The extensive functional genomics and analysis of 700,000 ESTs are available on an integrated Web-based browser (http://hoya.zool.kyoto-u.ac.jp/cgi-bin/gbrowse/ci) (Satou et al. 2005). Altogether, the assembly of two distinct *Ciona* genomes, the extensive EST database, the systematic characterization of gene function via antisense morpholino knock-down assays (e.g., Yamada et al. 2003), as well as the extensive spatial and temporal expression profiles for nearly one-fourth of all the predicted genes (e.g., Imai et al. 2004), have launched *Ciona* at the forefront of post-genome annotation and have opened the door to many new lines of research.

Genetics

Ciona offers the promise of "forward genetics," since the life cycle is relatively rapid (8–12 wk from fertilization to

F1 embryos) and it is possible to induce fertilization within hermaphrodites. Indeed, genetic screens have successfully identified mutations that disrupt notochord differentiation in *C. savignyi* (e.g., Nakatani et al. 1999), and there has been significant progress in establishing an infrastructure for facilitating further genetic analyses in both *C. intestinalis* and *C. savignyi*.

Using two different populations (British and Neapolitan) of *C. intestinalis*, Shungo Kano (Stazione Zoologica Anton Dohrn) constructed genetic maps, which were developed using an F1-pseudo-back-cross panel consisting of 50 individuals. He mapped a variety of amplified fragment length polymorphism (AFLP) markers, which fall into 14 linkage groups (identical to the number of chromosomes). It is now possible to produce sequence tags using the AFLPs to superimpose the genetic map onto physical chromosome maps. A similar effort is being conducted in *C. savignyi*, which displays even higher frequencies of polymorphisms in noncoding sequences than *C. intestinalis*. By using PCR-based restriction fragment length polymorphism (RFLP) markers where primers are anchored to adjacent exons, Matt Hill (Stanford University) reported successful construction of a genetic map of *C. savignyi* with an average recombination rate of 5 cM/Mb. We anticipate that accurate, high-resolution genetic maps will soon become available in both species of *Ciona*.

Miho M. Suzuki (Univ. of Edinburgh) reported that the *C. intestinalis* genome has 5-methylcytosine at ~20% of its CpG nucleotides. The detailed mapping of methylated CpG in the genome reveals that about one-third of all genes are methylated. *Ciona* provides an excellent opportunity to determine the role of differential methylation in gene regulation during development.

Mutagenesis

Wild populations of both *C. intestinalis* and *C. savignyi* provide an extensive source of mutants. Paolo Sordino (Stazione Zoologica Anton Dohrn) has screened recessive alleles by self-fertilization of individuals from wild populations of *C. intestinalis* in Italy. He has identified a variety of mutants with abnormal phenotypes that affect the morphogenesis and behavior of larvae. The genetic map that is being constructed for the Neapolitan population will facilitate the identification of the genes responsible for the mutant phenotypes. Meanwhile, Jason Tresser (University of California, Santa Barbra) described an ongoing ENU-based mutagenesis screen that has identified several patterning mutants in *C. savignyi*, including *vagabond* (*vag*). Mutations in *vag* cause defects in both the central and peripheral nervous systems. There is a block in the differentiation of palp epidermal neurons, which generate a signal required for entry into metamorphosis.

Yasunori Sasakura (Tsukuba University) reported successful germline transgenesis with the Tc1/*mariner* superfamily transposon, *Minos* (originally isolated from *Drosophila hydei*) (see Franz and Savakis 1991). In most marine invertebrates, metamorphosis is accompanied by dramatic morphological changes of larvae to produce a distinct adult body form. In ascidians, metamorphosis begins with the attachment of larvae to a substrate, followed by the rapid retraction of the tail into the trunk, and formation of adult organs within the trunk. One of the *Minos* insertional mutants, *swimming juveniles* (*sj*), undergoes metamorphic events in the trunk while larvae continue to swim with a fully functional tail. The *sj* mutant phenotype arises form the insertion of *Minos* into the promoter region of *Ci-CesA*, a gene encoding cellulose synthase (Sasakura et al. 2005).

The defining characteristic of all tunicates is the presence of an outer protective layer that is composed of tunicin, which is related to plant cellulose. It is thought that the genes responsible for the production of the tunic (or house) were acquired by an ancestral tunicate via horizontal gene transfer from bacteria more than 500 million years ago (Dehal et al. 2002; Matthysse et al. 2004). *sj* mutants fail to produce tunicin, and as a result, there is an arrest in metamorphosis. *Minos*-mediated germline transgenesis is also being used to create stable transgenic strains that express GFP in specific tissues and cell lineages.

Gene networks and organogenesis

There have been extensive gene duplication events in the vertebrate lineage since the time of the last shared ancestor of ascidians and vertebrates (Dehal et al. 2002). As a result, it is often difficult to determine the function of individual vertebrate patterning genes. For example, there are several Nkx2-5 genes in vertebrates, but only one such gene in *Ciona* (Davidson and Levine 2003). The combination of a streamlined, nonduplicated genome and simple cell lineages provides a powerful tool for the elucidation of the gene networks governing the formation of basic chordate tissues such as the notochord, neural tube, and heart.

Brad Davidson (University of California, Berkeley) presented an analysis of heart formation in *C. intestinalis*. Vertebrates contain two linked basic helix–loop–helix (bHLH) regulatory genes, *Mesp1* and *Mesp2*. *Mesp1* is essential for the specification of the cardiac mesoderm, while *Mesp2* controls somitogenesis (e.g., Takahashi et al. 2005). In *Ciona* there is a single *Mesp* gene that initiates heart formation. It is specifically expressed in the progenitors of the cardiac mesoderm at the 110-cell stage, the B7.5 blastomeres. These cells generate two lineages, an anterior lineage forming heart and a posterior lineage that forms the anterior tail muscles. Previous morpholino knock-down assays in *C. savignyi* have demonstrated that diminished *Mesp* function causes loss of the heart and the formation of supernumerary tail muscles (Satou et al. 2004).

Davidson showed that the expression of a hyperactivated form of Mesp (MespVP16) in the B7.5 lineage causes a dramatic phenotype: the transformation of anterior tail muscles into a beating heart. Normally, these muscle cells are subjected to programmed cell death during metamorphosis when the entire tail is resorbed and

destroyed. MespVP16 not only circumvents cell death during tail resorption but also transforms these cells into cardiomyocytes. Mosaic incorporation of the MespVP16 transgene into the posterior B7.5 lineage produces juveniles with two beating hearts: the normal heart that arises from the anterior B7.5 lineage and the transformed posterior lineage (Davidson et al. 2005). These studies provide the foundation for elucidating a complete gene network controlling heart cell differentiation.

There is considerable information regarding the gene network controlling notochord differentiation in the *Ciona* tadpole. The notochord is composed of a monolayer sheet of exactly 40 cells that rearrange to make a single rod of coin-shaped cells. The first step in notochord differentiation is the restricted expression of the *Brachyury* regulatory gene (*Ci-Bra*) in four "primary" and two "secondary" notochord progenitor cells at the 64-cell stage of embryogenesis (e.g., Takahashi et al. 1999). Studies in both *Ciona* and the distantly related ascidian *Halocynthia roretzi* provide a complete description of the regulatory events leading to the localized expression of *Ci-Bra* (and *Hr-Bra*). The localized zinc finger transcription factor Macho-1 is restricted to posterior vegetal blastomeres (Nishida and Sawada 2001), while the activation of β-catenin is restricted to anterior vegetal blastomeres (Imai et al. 2000). Macho-1 and β-catenin lead to the restricted expression of two key regulatory genes, *ZicL* and *FoxD*, as well as *FGF9* in the endoderm. Gaku Kumano (Osaka University) presented evidence that ZicL, FoxA, and FGF9 directly regulate *Hr-Bra* (and *Ci-Bra*) expression in the prospective notochord.

Bioinformatics of gene regulation

Ciona is an excellent experimental system to analyze the regulatory mechanisms of gene expression. A key strength is that whole-genome sequence assemblies are now available for two distantly related *Ciona* species, *C. intestinalis* and *C. savignyi*. Comparison of the genomes permits the identification of potential regulatory DNAs as conserved noncoding sequences. Sequence polymorphisms within individuals (a remarkable 4% in *C. savignyi* and 1.5% in *C. intestinalis*) also facilitate such studies. These methods have been used to identify a variety of tissue-specific enhancers in *Ciona*, including a cardiac-specific enhancer in the 5′-flanking region of the *Mesp* gene (Davidson et al. 2005).

It is also possible to identify shared sequence elements among coordinately regulated tissue-specific enhancers due to the ease of identifying large numbers of enhancers via electroporation. Arend Sidow and David S. Johnson (Stanford University) reported the identification of shared sequence features among a set of 20 different muscle-specific enhancers (Johnson et al. 2005). Similarly, Take Kusakabe (University of Hyogo) presented evidence that distinct combinations of regulatory elements could be identified in tissue-specific enhancers mediating restricted expression in muscles, neurons, or photoreceptor cells. He is creating a Web-based browser for the identification of *cis*-regulatory elements in the 5′-flanking regions of all known tissue-specific genes in the *Ciona* genome.

All known genes encoding sequence-specific transcription factors and components of cell signaling pathways have been extensively annotated. There are a total of 352 regulatory genes (including those that encode zinc finger transcription factors) and 109 signaling genes. cDNAs have been isolated for each of the 461 genes, and used to determine the exact timing and sites of expression, from fertilization through the onset of gastrulation (e.g., Imai et al. 2004). This information is freely available on an integrated Web browser (http://hoya.zool.kyoto-u.ac.jp/cgi-bin/gbrowse/ci).

It is now possible to obtain precise digital representations of gene expression profiles in the *Ciona* embryo. One of the most impressive reports at the meeting was by Patrick Lemaire (University of Marseilles). He described a three-dimensional computational software package, Aniseed (http://aniseed-ibdm.univ-mrs.fr/~ciona/ANISEED/index.php), which provides the exact shape, arrangement, and surface contacts between neighboring cells, from the 1-cell stage to the 44-cell stage (Tassy et al. 2005). The ultimate goal of the Aniseed program is to collate gene expression data, including gene networks, with the detailed behavior of individual cells, and groups of interacting cells, during complex morphogenetic processes such as tail elongation.

Cellular basis of development

Several talks focused on the role of subcellular components in morphogenesis. A key advantage of ascidians is the large size of individual blastomeres, and the ability to obtain high-resolution imaging of the cytoskeleton and organelles. Soon after fertilization, ascidian eggs exhibit two phases of ooplasmic segregation, which lead to the establishment of the embryonic axis and the segregation of maternal factors into appropriate territories in early embryos. Christian Sardet (Station Zoologique, Villefranche sur Mer) demonstrated the importance of the cortical endoplasmic reticulum (cER) in the localization and segregation of developmentally important mRNAs and proteins.

Convergence and extension of notochord cells drive tail elongation. These processes are now being studied in exquisite detail. Edwin Munro (University of Washington) tagged different subcellular compartments of notochord cells to visualize actin-based protrusive extension, actomyosin-based cortical contractility, and integrin- or cadherin-based cell adhesion. These studies led to the elaboration of a computational model that incorporated molecular studies of cell polarization, motility, and adhesion to explain notochord morphogenesis and tail elongation.

The notochord is also the source of important cellular signals that pattern neighboring tissues. Hiroki Takahashi (National Institute of Basic Biology, Japan) reported a fascinating finding: The notochord is essential for the patterning of the overlying central nervous system

(CNS). An ascidian homolog (Ci-Scale) of the *Drosophila Scabrous* gene (Mlodzik et al. 1990) is one of the downstream target genes of *Brachyury* that is essential for notochord differentiation. *Ci-Scale* is expressed exclusively in notochord cells of embryos at the tailbud stage, but its fibrinogen-like protein product becomes distributed underneath the CNS with fibril-like protrusions. Knock-down of *Ci-Scale* function resulted in the failure of convergent-extension movement as well as disruptions in CNS patterning, including axon guidance. The proper distribution of Ci-Scale proteins depends on Notch signaling delivered by the CNS, suggesting that cooperative interactions between Ci-Scale in the notochord and Notch in the CNS might be essential for CNS patterning in chordates. Perhaps similar interactions occur in vertebrates.

Nervous system and behavior

Ascidian larvae display distinct responses to gravity and light. For example, during the second half of larval life, tadpoles swim in response to diminished light intensity and stop swimming when the light is increased. One of the highlights of the meeting was the elucidation of the neuronal circuit underlying the swimming response.

Takeo Horie (University of Hyogo) presented the identification of glutamatergic, cholinergic, and GABAergic neurons in swimming tadpoles using specific antibodies. Glutamatergic neurons are present in the posterior sensory vesicle (SV) but not in the visceral ganglion (VG), which contain five left and right pairs of motor neurons. Cholinergic neurons located in the SV and VG extend posterior axons that innervate the anterior-most muscles in the larval tail. GABAergic neurons are present in the SV, VG, and anterior nerve cord. Two distinct pairs of bilateral GABAergic neurons in the anterior nerve cord extend anterior axons to contra-lateral cholinergic neurons. This circuit is responsible for the alternating contraction of each side of the tail.

Motonori Tsuda (University of Hyogo) described the visual pathway from photoreceptors to muscle cells. The pathway was traced by expressing a wheat germ agglutinin (WGA) transgene in the photoreceptor cells under the control of an arrestin enhancer. WGA transgene products in the photoreceptor cells were transported along their axons and *trans*-synaptically transferred to second neurons in the SV and ultimately transferred to the motor neurons. This imaging of the "swimming network" in *Ciona* represents an ancestral form of vertebrate motor systems, and provides the foundation for future behavioral studies.

Beyond *Ciona*: field studies and weird tunicates

One of the pleasures of working with ascidians is that they are members of a group of strange and diverse organisms that are used for a variety of field studies. A provocative talk was presented by Bernie Degnan (University of Queensland), who uses microarray assays for studying differential gene activity in diverse populations of the ascidian *Herdmania momus*. Two of the populations that were studied have adapted to very different environments in Australia and Hawaii. Significant differences were observed in the expression levels of 450 different genes.

There was considerable interest in the planktonic tunicate *Oikopleura dioica*, which lives in open oceans around the world. They possess several superb features for the genetic and molecular analysis of developmental processes. The complete life cycle is just 4–5 d, and embryogenesis is complete after 12 h (at 20°C). The genome of *O. dioica* is one of the smallest of all animal genomes: just 60 Mb (Seo et al. 2001).

Hiroki Nishida (Osaka University) reported an improved culture system using synthetic seawater, thereby making *Oikopleura* accessible to researchers around the world. Cristian Canestro (University of Oregon) described the patterning of the *Oikopleura* CNS. Based on the expression profiles of eight orthologs of vertebrate CNS regulatory genes, he proposed that tunicates lack the equivalent of the vertebrate midbrain. It remains to be seen whether the tunicates lost the midbrain from the last shared ancestor during the general degeneration of anterior tail structures (including the loss of Hox genes 7, 8, and 9), or if the midbrain is an innovation of the vertebrates.

In summary, ascidians have come of age and possess the full repertoire of methods for unraveling the most challenging problems in developmental biology. The integration of genetic and genomics methods, along with cell biological techniques, will yield comprehensive insights into morphogenesis, behavior, and ecological adaptation.

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