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The Ascidian as a Model Organism in Developmental and Evolutionary Biology

Minireview

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...at an extremely remote period a group of animals existed resembling in many respects the larvae of our present Ascidians, which diverged into two great branches—the one retrograding in development and producing the present class of Ascidians, the other rising to the crown and summit of the animal kingdom by giving birth to the vertebrata.

—Charles Darwin, *The Descent of Man* (1871)

Several years after the publication of *On the Origin of Species* in 1859, Darwin recognized the importance of the ascidian larva as a prototype of modern chordates. Despite their key phylogenetic position, ascidians were largely neglected by experimental biologists through most of the twentieth century. This fact is surprising since ascidian embryos provided the very first classical evidence that localized determinants control cell fate specification (Conklin, 1905). Furthermore, the ascidian larva represents an ideal model of early chordate development: it possesses the basic developmental and morphologic features of vertebrates but has the cellular and genomic simplicity of invertebrates. The purpose of this review is to describe the recent revitalization of interest in ascidians and their potential as a model system for developmental and evolutionary biology.

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What Is an Ascidian?

Ascidians, or sea squirts, are invertebrate chordates that belong to the earliest branch in the chordate phylum, the subphylum Urochordata or Tunicata (Figure 1A). Ascidian larvae possess a prototypical chordate body plan that includes a dorsal neural tube, an axial notochord flanked by muscles, and a ventral endodermal strand (Satoh, 1994; Figure 1B). Gastrulation and neurulation involve cellular rearrangements that are comparable to those seen in vertebrates, except that ascidian embryos are composed of just a few hundred cells whereas comparable vertebrate embryos contain many thousands of cells. The resultant motile larva (~1 mm long) is analogous to the amphibian tadpole. This larva ultimately undergoes metamorphosis into a sessile, filter-feeding adult (Figure 1C), hence Darwin's reference to ascidians "retrograding in development."

The larva of *Ciona intestinalis*, the most cosmopolitan ascidian species, has about 2,600 cells including only 36 muscle cells and 40 notochord cells. It has a remarkably simple central nervous system (CNS) with only 330 cells of which fewer than 100 are neurons, the remainder being glial cells (Meinertzhagen and Okamura, 2001). These figures place the ascidian larva on a level of cellular complexity comparable to that of *C. elegans* which has 959 somatic cells including 95 muscle cells and 302 neurons. As with *C. elegans*, ascidian cell lineages are invariant and have been completely defined for several tissues. The cell lineage of the *C. intestinalis* CNS has been traced through the penultimate cell division, and the connectivity of all neurons is being mapped (Meinertzhagen and Okamura, 2001 and references therein). Such an undertaking is simply not feasible in other chordates. The cellular simplicity of the ascidian larva is mirrored by its small, compact genome. The haploid genome size of *C. intestinalis* is ~160 Mb and contains ~15,500 genes (Simmen et al., 1998). This low figure, along with other

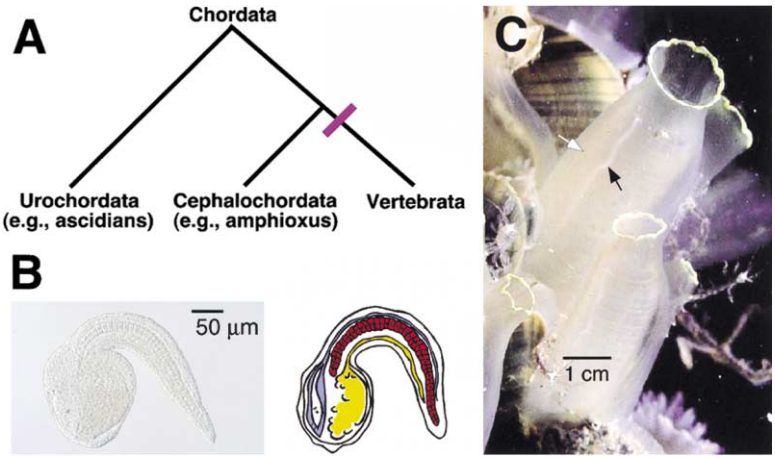

Figure 1. Chordate Phylogeny and the *Ciona* Life Cycle

(A) Chordate phylogenetic tree. Ascidians belong to the earliest branch in the tree, the subphylum Urochordata (Tunicata). The purple bar near the origin of the vertebrate clade represents the point at which the vertebrate genome underwent significant expansion in size and gene number.

(B) The ascidian larva. In the left panel is a photograph of a tailbud stage *Ciona intestinalis* larva (trunk to the left; tail to the right). The right panel shows a schematic depiction of a midsagittal section through the same larva. Note the dorsal nervous system (in blue) with the cerebral vesicle in the trunk region; the axial notochord (in red) which consists of a single-file row of 40 cells; and the underlying endodermal strand (in yellow). Paraxial muscles flanking the notochord are not visible in this plane of section.

(C) Adult sea squirt, *Ciona intestinalis*. The ascidian is hermaphroditic: the black arrow indicates the white spermatic duct and the white arrow indicates the orange-brown oviduct. (Photo courtesy of Arjan Gittenberger).

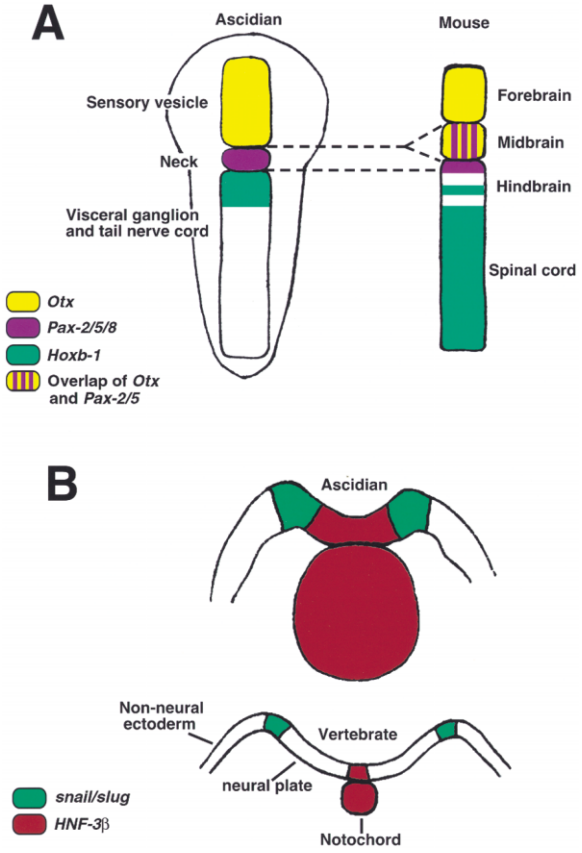

Figure 2. Conservation of Molecular Patterning in the Ascidian Larval Nervous System

(A) Gene expression along the anterior-posterior axis. Note the conservation of expression between mouse and ascidian. The name of the mouse gene is given in the figure; the corresponding ascidian homologs are *Hroth*, *HrPax-258*, and *HrHox1*, respectively. The "*Hr*" prefix in the ascidian gene names stands for "*Halocynthia roretzi*" the species in which these studies were performed. (Adapted from Wada et al., 1998.)

(B) Gene Expression along the dorsal-ventral axis. The ascidian *HNF-3*β homolog (*Ci-fkh*) is expressed both in the notochord and the ventralmost cell of the neural tube as in vertebrates. The ascidian *snail/slug* homolog (*Ci-sna*) is expressed at the lateral border of the neural plate, the cell population from which the neural crest arises in vertebrates. Note that only the names of the vertebrate homologs are given in the figure. (Adapted from Corbo et al., 1997b.)

evidence such as the presence of single-copy *Hox* genes (Di Gregorio et al., 1995), confirms that the ascidian genome predates the expansion in gene number that occurred in vertebrates (Figure 1A).

Recent studies indicate that the ascidian larva and vertebrate tadpole demonstrate extensive evolutionary and molecular homologies. For example, many of the same genes are selectively expressed in the ascidian and vertebrate notochord (e.g., Takahashi et al., 1999). There is also striking conservation in the patterning of the ascidian and vertebrate nervous systems (Corbo et al., 1997b; Wada et al., 1998). The ascidian CNS exhibits sequential patterns of *Otx*, *Pax*, and *Hox* expression along the anterior-posterior axis that correspond to the patterns seen in vertebrates (Wada et al., 1998; Figure 2A). Dorsal-ventral patterning is also conserved, even though the ascidian's caudal neural tube is composed of just four cells when considered in cross-section (Figure 2B). The ventralmost cell expresses *HNF-3*β and collagen 2A1, which suggests homology with the vertebrate floor plate (Corbo et al., 1997b). Moreover, the cells at the lateral border of the neural plate express *snail/slug* and *Pax-3/7* homologs, similar to the expression profiles seen in the vertebrate neural crest (Corbo et al., 1997b; Wada et al., 1998 and references therein). Although ascidians do not have functional neural crest cells, the striking conservation in the expression patterns of these ascidian homologs raises the possibility of the existence of a molecular precursor to neural crest in protochordates.

Analysis of Gene Function in Ascidians

The simplicity of the ascidian genome has important implications for the study of gene function. First, many genes present in multiple copies in vertebrates appear to have a single homolog in ascidians. Hence, the ascidian genome appears to contain the basic chordate genetic "toolkit," and thus represents an ideal reference point for the expanded vertebrate genomes. Furthermore, the single-copy nature of the ascidian genome is likely to facilitate identification of the function of novel proteins as it obviates the problem of so-called "genetic redundancy" that has bedeviled many studies in vertebrates.

In fact, ascidians are amenable to genetic analysis. Ascidians of the genus *Ciona* have a relatively short life cycle (1–2 months), are small and can be maintained at high density, have sperm which can be cryopreserved for maintenance of mutant strains, and most importantly are hermaphroditic and self-fertile: each adult animal carries both sperm and egg which can be mixed to produce viable offspring. This latter fact permits rapid screening for recessive mutant phenotypes in only two generations. Preliminary genetic screens have been carried out in *C. intestinalis* and a sister species, *Ciona savignyi*, resulting in the isolation of a range of interesting mutants (Moody et al., 1999; Sordino et al., 2000). The remarkable cellular simplicity of the ascidian larva with its paucity of tissue types, its near transparency, and rapid development (18 hr from fertilization to terminal differentiation), greatly facilitate the screening for mutant phenotypes at single-cell resolution.

Gene Regulatory Networks

Another important consequence of the compactness of the ascidian genome is the ease with which *cis*-regulatory elements can be identified. A number of tissue-specific enhancers that direct expression in the notochord, tail muscles, and CNS have been characterized in detail (e.g., Figure 3). In all cases so far examined, these enhancers are located in the first few kilobases upstream of the transcriptional start site, whereas comparable enhancers are sometimes located more than 10 kb 5′ or 3′ of homologous genes in vertebrates (e.g., notochord and CNS enhancers in the *HNF-3*β gene; Nishizaki et al., 2001; Di Gregorio et al., 2001). The detailed characterization of these enhancers has been greatly facilitated by the advent of simple electroporation procedures for incorporating transgenic DNA into developing *Ciona* embryos (Corbo et al., 1997a). This method is faster, simpler, and more efficient than microinjection and permits the simultaneous transformation

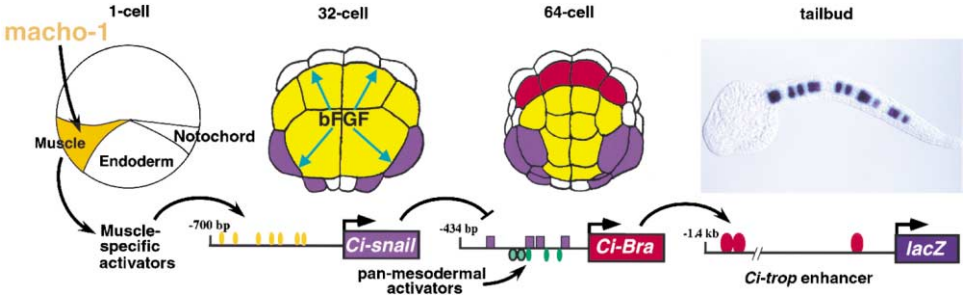

Figure 3. Gene Regulatory Network Governing Notochord Differentiation

1-cell stage (lateral view; vegetal pole down; animal pole up): localized maternal *macho-1* mRNA probably represents the classical ascidian muscle determinant (in orange); muscle-specific transcriptional activators, possibly induced by macho-1, activate *Ci-snail* by binding to upstream E box motifs (for details see Erives et al., 1998). 32-cell stage (vegetal view): *Ci-snail* expression begins in muscle and trunk mesenchyme precursors (in purple); bFGF (and possibly other factors) from the endoderm (in yellow) induces the expression of pan-mesodermal activators of *Ci-Bra* throughout the presumptive mesoderm, but *Ci-Bra* expression is excluded from muscle/trunk mesenchyme by the presence of the *Ci-snail* repressor in these tissues (for details see Fujiwara et al., 1998). 64-cell stage (vegetal view): *Ci-Bra* expression (in red) begins in notochord precursors (secondary notochord lineage omitted for simplicity). Tailbud stage: Ci-Bra activates expression of a battery of downstream genes involved in differentiation of the notochord including *Ci-trop*. The photomicrograph depicts a tailbud larva that was electroporated at the 1-cell stage with a transgene consisting of *lacZ* fused to the *Ci-trop* enhancer which contains clustered Ci-Bra binding sites (Di Gregorio and Levine, 1999). Note the notochord-specific pattern of X-gal staining. Not all notochord cells express *lacZ* due to mosaic incorporation of the transgene. (The photomicrograph is adapted from Di Gregorio and Levine, 1999.)

of hundreds, even thousands, of synchronously developing embryos.

Electroporation has also been used to assess gene function, such as the role of *Brachyury* in the development of the notochord (Takahashi et al., 1999). Normally the *Ciona Brachyury* homolog, *Ci-Bra*, is specifically expressed in the notochord (Figure 3), but when its coding sequence is attached to the enhancer of the *Ciona HNF-3β* gene, it is efficiently misexpressed in the endoderm and CNS. This ectopic expression causes a severe mutant phenotype, wherein endoderm precursor cells are partially transformed into notochord. Subtractive hybridization screens were conducted to identify genes that are overexpressed in mutants as compared with normal larvae. Nearly 40 notochord-specific genes were identified, and at least one, *Ci-tropomyosin-like*, has been shown to be directly activated by Ci-Bra (Di Gregorio and Levine, 1999; Figure 3). Thus, the regulatory network underlying notochord formation in ascidians appears to be "shallow" in that genes involved in terminal phases of differentiation (e.g., cytoskeletal genes) are direct targets of the selector genes involved in fate determination (i.e., *Ci-Bra*). This fact may reflect the rapid development of the ascidian larva and again underscores the basic nature of the ascidian system as compared with vertebrates, which exhibit "deeper," multitiered hierarchies of gene regulation.

Nearly a century after Conklin first demonstrated the existence of a localized "maternal determinant" for muscle formation in the cytoplasm of ascidian eggs (Conklin, 1905), Nishida and Sawada (2001) have cloned a gene, *macho-1*, which appears to represent the classical "muscle determinant" and therefore lies at the top of the gene network controlling muscle formation. *macho-1* encodes a zinc-finger transcription factor whose mRNA is localized to a swath of cytoplasm in the posterior, vegetal regions of the fertilized egg that coincides with the muscle-determining "yellow crescent" described by Conklin (Figure 3). Ectopic expression of *macho-1* causes the formation of supernumerary tail muscle cells (Nishida and Sawada, 2001), demonstrating that the gene is sufficient for determination of muscle fate.

macho-1 also plays an indirect role in restricting notochord fate to a subpopulation of mesodermal cells. Nishida and colleagues have shown that an inductive interaction between presumptive endoderm and mesoderm at the 32-cell stage is required for determination of notochord fate in mesodermal precursors (Kim et al., 2000 and references therein). This induction, which may be mediated by bFGF, has the potential to induce *Ci-Bra* expression in most or all of the adjacent mesoderm cells, both the presumptive tail muscles and notochord, but *Ci-Bra* expression is restricted to the notochord by specific repressors in muscle precursors (Figure 3). One such repressor is *Ciona snail*, which has been shown to specifically repress *Ci-Bra* expression by binding to its enhancer (Fujiwara et al., 1998). It is conceivable, but not currently known, that macho-1 activates *Ci-snail* in the muscle lineage.

Altogether, the arsenal of tools available for the analysis of ascidian embryogenesis has provided an outline of the gene regulatory network governing notochord differentiation, as summarized in Figure 3. This network extends from localized determinants in the egg and culminates in the cellular morphogenesis of the tail. The application of genomic technologies should permit the characterization of complete regulatory networks underlying formation of not just the notochord, but also other key chordate tissues, such as muscle, endoderm, and CNS.

The Ascidian as a Prototype of Vertebrate cis*-Regulation*

The central role of changes in *cis*-regulatory architecture as a mechanism for evolutionary modification of animal body plans is widely recognized (Carroll et al., 2001). However, in contrast to protein coding sequences, noncoding *cis*-regulatory DNA is poorly understood due to the technical difficulties associated with detailed in vivo

enhancer analysis in vertebrates (Davidson, 2001). Unlike genes, there have not been sufficient studies of *cis*-regulatory elements to be able to categorize them into "families" or for that matter to even describe a basic grammar governing the manner in which individual *cis*-regulatory elements are assembled from their component parts (i.e., activator and repressor binding sites). This fact is all the more dismaying considering that ~98% of the human genome appears to be noncoding and is likely to be very rich in *cis*-regulatory information. In this context, we expect the ascidian larva to prove its worth. No other model system, either vertebrate or invertebrate, affords a more rapid or facile assay for the genome-wide identification and characterization of *cis*-regulatory elements.

It is possible to envision a complete, functional annotation of the ascidian genome in which tissue-specific enhancers are mapped for every gene, and the regulation of every enhancer is understood at the level of interacting regulatory factors acting downstream of defined cell signaling pathways. Progress toward this goal will be greatly hastened upon the completion of current whole-genome sequencing projects for *Ciona intestinalis* (Joint Genome Institute, USA and the National Institute of Genetics, Japan) and *Ciona savignyi* (MIT/Whitehead Institute). Since the ascidian larva predates the genomic expansion and dramatic increase in morphologic complexity that occurred in the vertebrate clade, we can infer that its *cis*-regulatory architecture may more closely reflect that of the ancestral chordate. For this reason, a detailed understanding of the ascidian transcriptional regulatory network will represent a critical model of the basic structure from which the more complex architectures of vertebrates evolved. Despite its "retrograde" development into a sea squirt, the ascidian larva may well rise to the crown and summit of the genomic era by serving as a template for decoding vertebrate genomes.

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