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REVIEW

Genetic perspectives on the ascidian central nervous system

A Locascio*, F Ristoratore*, A Spagnuolo, L Zanetti, M Branno

Cellular and Developmental Biology Laboratory, Stazione Zoologica "Anton Dohrn", Villa Comunale, Napoli, Italy

*Equal contribution

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Abstract

In 2002, date of publication of the *Ciona intestinalis* genome, ascidians entered the post-genomic era. This tool had a fundamental role and has become the starting point for a series of new functional and genomic studies. Recently, great efforts have been done to characterize the genetic cascades of genes having a key role in early embryonic development and to draw the regulatory networks in which they are involved. In this review, we focused our attention on the last advances obtained in the attempt to clarify the complex molecular events governing ascidian central nervous system development with a special interest for anterior neural and sensory structures. We discussed the more recent theories on its early induction and late regionalization. In particular, we used some conserved genes fully or partially characterized as examples to compare ascidian and vertebrate central nervous system (CNS).

By integrating the various results obtained with microarray, morpholino loss of function and promoter analyses, we showed that many progresses have been done to unravel the gene networks controlling early CNS induction and formation. Unfortunately, fewer advances have been done in the identification of the regulatory cascades controlling late CNS regionalization and sensory organs differentiation. Some results are discussed to point out the importance of fully characterizing also these specific regulatory cascades.

Key words: ascidian; nervous system; genetic pathway; genome; regulatory network

Introduction

Situated at the base of the chordate lineage, ascidians possess many features shared with vertebrates; the larval form, in particular, has been recognized as evolutionarily significant because it reveals features of the early evolution of the vertebrate body plan. Due to their relative simplicity and their crucial phylogenetic position, ascidians have the unique potential to illuminate molecular mechanisms underlying the ancestral body plan from which modern Chordates diversified.

In particular, the larval nervous system of *Ciona intestinalis,* consisting of a brain vesicle, a visceral ganglion and a tail nerve chord, is simple but well differentiated. The *Ciona* central nervous system (CNS) can thus be viewed as a miniature prototype of the chordate brain and represents an exciting experimental model to understand ancestral features of the chordate nervous system, its development and physiology.

Corresponding author:
Annamaria Locascio
Cellular and Developmental Biology Laboratory
Stazione Zoologica "Anton Dohrn"
Villa Comunale, 80121, Napoli, Italy
E-mail: anny@szn.it

Thanks to their transparent embryos, ascidians have been a model system for embryological studies for over a century. A number of new methodologies, developed in recent years and ranging from molecular, genomic, and physiological approaches, are greatly contributing to the knowledge of the complex regulatory networks underlying ascidian CNS development.

The sequencing of *C. intestinalis* (Dehal *et al*., 2002) and *Ciona savignyi* (http://www.broad.mit.edu/annotation/ciona/) genomes and the accumulation of molecular resources, that rival those available for fruit flies and mice, introduced ascidians into the post-genomic era.

Ciona embryos are readily amenable to experimentation in the laboratory and in vitro fertilization can produce thousands of synchronously dividing embryos that develop rapidly. Ascidians are hermaphrodites, and the ability of *Ciona* to self-fertilize provides a rapid means for identifying recessive zygotic mutations (Moody *et al*., 1999; Sordino *et al*., 2001). A screening for mutations naturally occurring in a large percentage of wild *Ciona* populations (Sordino *et al.*, 2008), identified more than 40 % of the

mutations lead to morphological alterations at level of brain and sensory vesicle formation. This natural genetic polymorphism constitutes a valuable source of phenotypes for studying embryonic development. Developmental mutants have also been obtained in both *Ciona* species through chemical mutagenesis (Moody *et al.*, 1999; Sordino *et al.*, 2000; Sordino *et al.*, 2001) and, more recently, through insertional mutagenesis using the Minos transposable element (Sasakura *et al.*, 2003). Additionally, recently generated stable transgenics expressing GFP represent useful tools for investigations on mechanisms of tissue formation during embryogenesis and morphogenesis. Both mutagenesis and stable transgenesis in *Ciona* require the rearing of animals in the laboratory for several generations. This requirement has been assisted by the development of new culture techniques and maintenance protocols (Cirino *et al.*, 2002; Hendrickson *et al.*, 2004), and recently improved by introducing the use of closed, recalculating sea water systems to permit *Ciona* culture in inland laboratories (Joly *et al.*, 2007).

Using electroporation, transient transgenesis is successfully performed in ascidians allowing characterization of *cis*-regulatory DNAs (Alfano *et al.*, 2007; Corbo *et al.*, 1997; Erives *et al.*, 1998; Ristoratore *et al.*, 1999). The wide number of tissue-specific enhancers currently available allows both the production of mutant phenotypes, *via* ectopic expression of regulatory genes (Spagnuolo and Di Lauro, 2002; Takahashi *et al.*, 1999), and disruption of gene activity through the overexpression of dominant negative proteins (Corbo *et al.*, 1998). Injection of morpholino antisense oligonucleotides (Satou *et al.*, 2001) provides an alternative method for the analysis of loss of gene function. The use of comprehensive microarrays has revealed extensive temporal patterns of gene activity (Azumi *et al.*, 2007a) and assisted in the determination of spatial patterns of gene expression within individual blastomeres in sequentially staged embryos (Yamada *et al.*, 2005). Recently, a simple method allowing the maintenance of dissociated cells from *Ciona* in primary cultures has been developed (Zanetti *et al.*, 2007). Cells that conserve their functionality have been successfully cultured, opening the possibility that this method could be used in a wide range of experiments in this animal model. In particular, this method allowed the identification of two types of neurons resembling motorneurons and large eminens cells (Imai and Meinertzhagen 2007a, b). Taking into account that the ascidian nervous system is largely inaccessible to neurophysiological studies because of the tough outer tunic of the larvae, primary cell culture represents a useful system to overcome these limitations. Collectively, all these technical advancements in *Ciona* provide powerful tools for the study of the gene networks controlling chordate development.

Here we summarise the more recent advances on the genetic mechanisms underlying the development of nervous system structures, highlighting the similarities as well as the differences observed across chordates. We will focus primarily on *C. intestinalis* and, where appropriate, we discuss also findings from other ascidian species.

Ascidian CNS in chordate evolution

Six years have passed from the publication in Science of the *C. intestinalis* genome sequence (Dehal *et al.*, 2002) and since then much progress has been made in the knowledge of both genomic and developmental processes. The genomes of another *Ciona* species, *C. savignyi* (http://www.broad.mit.edu/annotation/ciona/) and of the appendicularian *Oikopleura dioica* (http://www.genoscope.cns.fr/spip/Oikopleura-dioica-whole-genome.html) have been sequenced and assembled. Advances in genome annotation (Ensembl), FISH analysis (Shoguchi *et al.*, 2008) and identification of AFLP markers (Kano *et al.*, 2006) greatly contributed to the construction of *Ciona* chromosomal maps. Shoguchi *et al.* (2008) mapped about 82 % of the genome sequence information on all arms of the *Ciona* chromosomes. What emerged from these studies is that regulatory genes and their targets, belonging to the same developmental gene networks, are distributed over all fourteen chromosomes. Thus, genes whose genomic regulation and functions are interconnected do not need physical clustering to be coordinately activated. All together, this new piece of information makes ascidians a very powerful model system for functional genomic studies.

Among the main novelties there is the new ascidian phylogenetic position as sister group to the vertebrates, a position long thought to be held by the Cephalochordates. Taking advantage of the genome sequence of the tunicate *Oikopleura dioica*, a set of 146 genes from 14 deuterostome species have been aligned and phylogenetically analyzed. The results suggest that tunicates do not represent the earliest chordate lineage, as previously inferred, but in fact they are the closest living relatives of vertebrates (Delsuc *et al.*, 2006).

Recently the *Ciona* genome release has been complemented by large scale cDNA and EST projects accompanied by expression analyses (http://ghost.zool.kyoto-u.ac.jp; Imai *et al.*, 2004; Satou and Satoh, 2005). cDNA and oligo-DNA microarrays allowed the construction of new urochordate expression map databases (Azumi *et al.*, 2007b; Yamada *et al.*, 2005) and detailed expression profiles are now available for most of the genes encoding cell signaling and regulatory proteins.

A three dimensional computational software organizes and integrates gene expression data with the complex single cell developmental programs (http://crfb.univ-mrs.fr/aniseed/). A three dimensional ascidian body atlas database shows interactive developmental tables of 26 newly defined embryonic stages, accompanied by cell lineage descriptions and time laps photos (http://chordate.bpni.bio.keio.ac.jp/faba/1.2/top.html).

All these data, together with the key position of ascidians at the base of vertebrate origin, give this system the power to clarify the complex gene networks governing Chordate evolution and

vertebrate development.

The evolution of the CNS and in particular of its anterior structures is a crucial point in the chordate lineage. Vertebrates have a complex, segmented and highly organized CNS that evolved from the same chordate ancestor of ascidians. Many complex and still poorly defined regulatory networks governing nervous system development and differentiation can be clarified by taking advantage of recent progress in functional genomic studies and the compact and non redundant genome of ascidians.

The ascidian larva is composed of only ~2600 cells of which about 330 cells constitute the CNS. Following the basic chordate body plan, neurulation gives rise to a sensory vesicle of ~215 cells, including two pigmented sensory organs and, in sequence along the antero-posterior axis, to the neck, the visceral ganglion and dorsal hollow nerve cord (Nicol and Meinertzhagen, 1991).

Despite the evident morphological differences between ascidian and vertebrate CNS structures, there are clear homologies in nervous system patterning as deduced from the expression patterns of many developmental genes.

The *Otx* gene defines the forebrain of vertebrates, and marks also the ascidian larva sensory vesicle (Fig. 1). This region has been compared to the vertebrate rostral CNS and is considered analogous to the forebrain (Wada *et al.*, 1998).

A series of vertebrate genes, *FoxH1*, *Nkx2.1* and *Otp*, are markers of the developing hypothalamus and specifically are involved in cell fate restriction, hypothalamus primordium delineation and neuroendocrine hypothalamic nuclei differentiation. The ascidian homologues (*Ci-FoxHa*, *Ci-Nkx2.1* and *Ci-Otp*) were found to be expressed in regionalized patterns within the *Ciona* ventro-lateral sensory vesicle. These data suggest a possible correspondence between these vertebrate and ascidians structures (Moret *et al.*, 2005). Supporting this suggestion of homology, the coronet cells on the left side of the sensory vesicle in ascidians have been structurally compared to the cells of saccus vasculosus in the vertebrate hypothalamus (Katz, 1983). Similarities can also be observed at the level of CNS patterning, exemplified by the regionalized expression domains of *Hox* genes. Nine *Hox* genes have been identified in the *Ciona* genome (Dehal *et al.*, 2002; Spagnuolo *et al.*, 2003), which have undergone a significant reorganization, including rearrangement of the gene positions, dispersion, and breakage on separate chromosomes. Despite the extensive shuffling of *Ciona Hox* gene cluster, some of the gene members maintain a coordinated expression in the larval CNS. *Ci-Hox1*, *3*, *5* and *10*, indeed, show a restricted and colinear expression profile along the antero-posterior axis at the level of the visceral ganglion and nerve cord, which are considered homologous to vertebrate hindbrain and spinal cord respectively (Gionti *et al.*, 1998; Ikuta *et al.*, 2004; Locascio *et al.*, 1999).

The regionalized expression patterns in ascidians of specific markers of vertebrate forebrain, midbrain, mid-hindbrain boundary (MHB) and hindbrain, such as *Otx*, *Pax2/5/8*, *En*, and the *Hox* genes, suggest a tripartite organization of the ascidian CNS. In particular, the sensory vesicle, marked by the *Otx* gene, is considered homologous to the vertebrate fore-midbrain; the neck region marked by *Pax2/5/8* and *En* is considered to be homologous to the vertebrate MHB; finally the visceral ganglion marked by *Hox* genes corresponds to the vertebrate hindbrain (Fig. 1) (Imai *et al.*, 2002; Wada *et al.*, 1998).

More recently, the presence in *Ciona* of a midbrain homologous region has been reconsidered based on a comparative analysis of the expression pattern of *Dmbx*, a marker of the midbrain in vertebrates (Takahashi and Holland, 2004). Takahashi and Holland (2004) demonstrated that the ascidian *Dmbx* gene is expressed only in the visceral ganglion, in a region that is posterior to the *Pax2/5/8* expression territory (MHB homologue) and coincident with rostral limit of *Hox* gene territories (hindbrain homologue). The presence of the *Ciona Dmbx* gene only in the visceral ganglion could correspond to the expression of vertebrate *Dmbx* in the hindbrain (Fig.1), thus reinforcing visceral ganglion homology with the vertebrate hindbrain and suggests that a midbrain homologue is missing in *Ciona*. Data from amphioxus, where no *Dmbx* expression is observed in the neural tube (Takahashi and Holland, 2004), further support the notion that the midbrain is a novelty that evolved specifically in the vertebrate lineage.

Recently, a detailed analysis in *Ciona* at various embryonic stages of the dynamic expression profiles of *CiOtx*, *Pax2/5/8*, *CiFgf8/17/18*, *En* and *Hox* genes in part supports the tripartite model but in part indicates that also a dipartite model, lacking the MHB region, may be consistent (Ikuta and Saiga, 2007). The expression profiles of *Pax2/5/8* and *En* in the neck region and the presence of an intervening gap between *Otx* and *Hox* gene expression territories in larval stages support the tripartite organization (Fig.1). Expression of *Fgf8/17/18* seems to be in contrast with this model. In vertebrates, *Fgf 8* is expressed in the MHB and play a pivotal role in the organizer activity (the capacity to change the fate of surrounding tissues when transplanted in other CNS territory) of this region (Liu and Joyner, 2001). In *Ciona*, the absence of *Fgf8/17/18* expression in the neck region and its presence only more posteriorly in the visceral ganglion (Fig.1) could indicate that ascidians possess an ancestral MHB lacking organizer activity. On the other hand, the lack of a gap between *Otx* and *Hox* domains at earlier stages of development supports a dipartite organization. Dufour (2006) proposed a third model based on *CiPhox2* expression at larva and adult stages. In vertebrates Phox2 genes (*Phox2a* and *Phox2b*) mark the hindbrain and define cranial motorneurons of the branchiovisceral class. In ascidians, *CiPhox2* is restricted to the neck region at larva stage and will

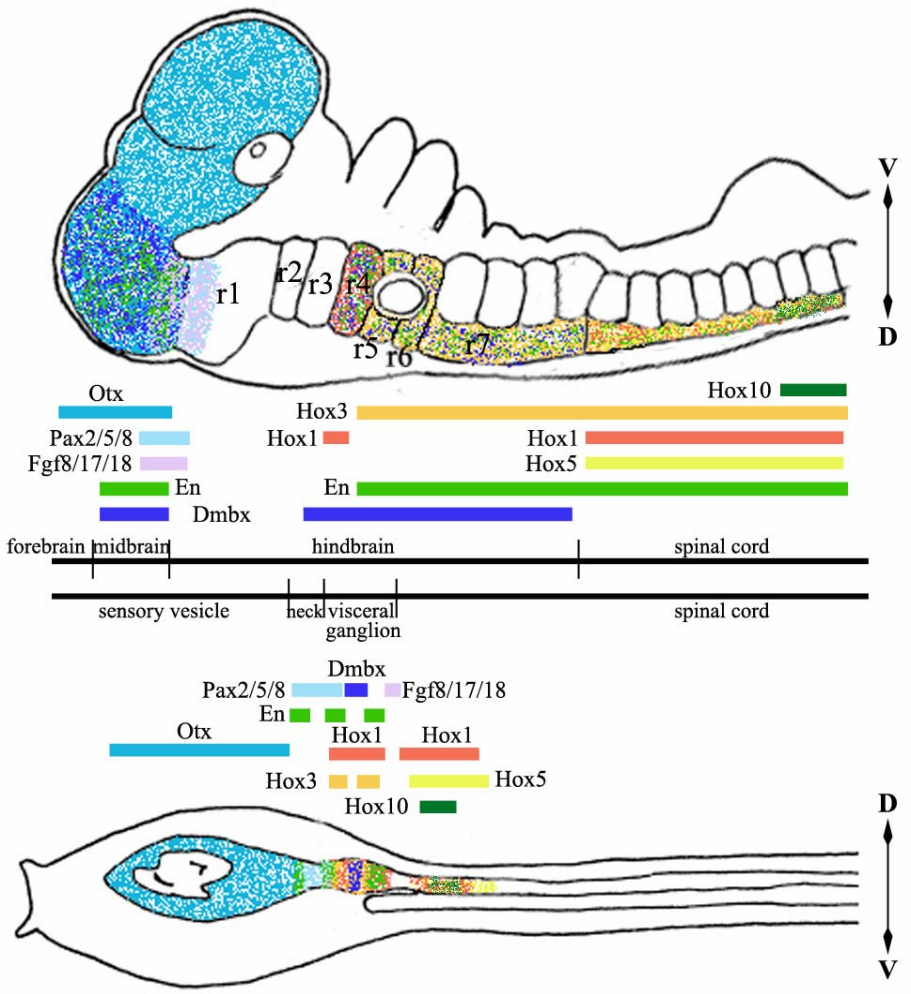

Fig. 1 Comparison of gene expression domains in the central nervous system of ascidians and vertebrates. Homologous genes are indicated by the same colours. The most anterior regions are marked by *Otx* (blue) genes and correspond to the sensory vesicle in ascidians and the forebrain and midbrain in vertebrates. *Dmbx* gene (dark blue) is expressed in the midbrain only in vertebrates, while it is present in the hindbrain (visceral ganglion) in both chordate organisms. Expression of *Pax2/5/8* (light blue) and *En* (green) in the intervening region between *Otx* and *Hox* genes (*Hox1*, red; *Hox3*, orange; *Hox 5*, yellow; *Hox10*, dark green) is common to both vertebrates and ascidians. *Fgf8/17/18* (lilac) is a marker of the MHB region, at the junction between midbrain and hindbrain, in vertebrates while it is expressed more posterior in the visceral ganglion in ascidians. D, dorsal; V, ventral.

give rise to the adult motorneurons located in the cerebral ganglion. These results, together with *Hox* genes expression profiles, suggested a possible homology of the posterior half of the neck with vertebrate hindbrain and of the visceral ganglion with spinal cord (Dufour *et al*., 2006). Unfortunately these controversial data, together with the absence in ascidian genome of another marker of vertebrate MHB, the *Gbx* gene (Castro *et al.*, 2006), make it difficult to clearly establish which model most appropriately reflects the ascidian condition and thus, the appearance during Chordate evolution of the MHB organizer. Gene expression analyses in additional deuterostome organisms (eg. Hemichordate) could help to elucidate the ancestral situation of the basic patterning of CNS and the subsequent evolutionary steps that led to the differentiation of the highly complex vertebrate nervous system.

CNS regulatory networks

Ascidian neural specification is organized via conserved chordate gene networks. These networks have been expanded during vertebrate evolution to create more complex neural structures. In ascidian embryos, blastomere divisions are initially synchronous, and become partially asynchronous later in development. Invariant cleavages and cell lineages give rise in *Ciona* to about 330 neural cells, of which about 2/3 form the sensory vesicle, 47 the visceral ganglion and 95 the caudal nerve cord. At the 110 cell stage, most of the blastomere fates have been already determined; descendents of the

a-line form the anterior neural structures, while the neural tube derives from the b8.19 and A7 cell lines (Cole and Meinertzhagen, 2004).

It was already known that Fgf9/16/20 has a fundamental role in inducing anterior neural differentiation in the a-line, while Nodal and Erk signaling are involved in caudal neural tube formation (Imai *et al.*, 2006; Lemaire *et al.*, 2002). Imai *et al.* (2006) contributed much to our knowledge of gene networks involved in *Ciona* neurogenesis. Large scale morpholino assays and subsequent characterization by RT-qPCR of altered gene expression in the mutant phenotypes permitted the establishment of a series of regulatory relationships at the level of a single cell. In particular, they focused their attention on regulatory genes specifically localized in the blastomeres determined to acquire a particular cell fate. The analysis and comparison of significant changes in the expression profiles of genes permitted the description of the early networks that lead to the induction of different tissues and to the establishment of restricted cell fates (Imai *et al.*, 2006). Unfortunately, morpholino studies do not permit the observer to unequivocally distinguish between direct and indirect target genes. The combination of morpholino "loss of function studies" of genes of interest together with promoter analyses of their candidate downstream genes has a great potential for the elucidation of regulatory networks and reconstruction of the precise genetic cascades leading to a specific tissue differentiation. Ascidians represent indeed a very suitable model system to perform regulatory element assays given their compact genomes and the frequent location of minimal promoters approximately <1 kb upstream of the transcription start sites of the genes of interest. Moreover, the opportunity to compare specific genomic fragments between closely related species represents a further advantage that these organisms offer. Additionally, electroporation is a relatively simple and efficient method that gives hundreds of transgenic embryos (Corbo *et al.*, 1997).

An interesting example of the results that can be achieved combining loss of function and gene promoter studies is offered by the reconstruction of the neural induction and *Otx* genetic cascade during early embryonic development.

Ascidian ectodermal induction starts at the 8-cell stage when *Ci-GATAa* expression is repressed in vegetal territories by β-catenin accumulation, and thus restricted to the animal hemisphere. At 8-16 cell stage, *Ci-GATAa* activates pan-animal pole genes, such as *Ci-Fog*, while *Fgf9/16/20* is active as a neural inducer in the a-line. At the 32 cell stage brain specification begins with the activation of the *Otx* gene through Fgf9/16/20 signaling. Studies of the *Ci-Otx* promoter revealed the presence of seven *Ci-GATAa* sites for animal activation and two *Ets* binding sites for its restriction to neural cells induced by FGF (Bertrand *et al.*, 2003; Rothbacher *et al.*, 2007). In particular, Fgf9/16/20 activates *Otx* and *Dmrt1* and both of them, on their own, activates transcription factor genes such as *Six1/2* and *Six3/6* (Imai *et al.*, 2006).

Nodal is a signaling molecule of the TGFβ factor superfamily, and acts as an organizing signal in the development of the nerve cord. At the 32 cell stage, FGF9/16/20 activates also *Nodal* whose expression is restricted to the b6.5 cells through the activity of both SoxC and FoxA. Nodal activates *Msxb*, *Pax3/7*, *Snail*, *Delta-like* and *Chordin* in the b6.5 cell line and induces roof nerve cord differentiation. In the A7.8 lineage, Nodal induces *Snail*, *Delta-like* and *Neurogenin* to form the lateral ependymal cells of the nerve cord (Imai *et al.*, 2006).

These studies have extended our understanding of the first phases of neural induction and formation; on the other hand, our knowledge on the gene networks acting later in development, during nervous system regionalization and differentiation, is still very poor.

In most cases the roles of genes temporally expressed at various stages of development have been studied in detail only at early embryonic stages by morpholino loss of function experiments; their roles at later stages are still largely unknown. Only few genes specifically expressed later in development, and potentially involved in neural structure differentiation, have been studied in detail. In most cases their function, and the genetic cascades in which they are involved, are only partially defined and remain unconnected to other known regulatory networks. To analyze the function of genes involved in late events of CNS differentiation, a morpholino approach may give results difficult to interpret; studies of their transcriptional regulation could be more informative on the regulatory networks in which these genes are involved.

Here we offer three examples of genes expressed in the CNS at late stages of *Ciona* development; dissection of their promoter regions has already given some clues to the elements controlling their expression. Further studies are needed to identify their upstream regulators in order to define some steps of the genetic circuits at the bases of CNS regionalization.

Hox genes represent a good example of conserved regulatory genes involved in late CNS regionalization. Several ascidian *Hox* genes show conserved expression patterns, indicating a role in patterning the antero-posterior axis, however most studies treat their expression profiles, genomic, and chromosomal organization adding little functional data. The study of *CiHox3* promoter, the only one deeply analysed, led to the characterization of an 80-bp element found to be sufficient to recapitulate the endogenous expression pattern of *CiHox3* (Locascio *et al.*, 1999). Unfortunately this sequence did not show any likely recognition sequence for known transcription factors, this requires a deeper bioinformatics analysis to identify putative binding sites. The 80-bp *CiHox3* promoter fragment however served to address whether *Hox3* regulatory elements have been conserved during Chordate evolution. The 80-bp *CiHox3* promoter fragment, when tested in mouse embryos, was unable to reproduce any neural-specific expression in transgenic mice, thus suggesting that the elements regulating *Hox3* expression are not conserved

between *Ciona* and mouse. Nevertheless, it is interesting to note that, when a slightly larger enhancer fragment was tested, a reproducible segmental pattern of expression in the mouse hindbrain was obtained, suggesting that some elements acting in *Ciona* are recognised by mouse transcriptional machinery (Locascio *et al.*, 1999). These early interesting results give rise to the possibility of identifying *Hox* specific enhancer elements responsible for antero-posterior regionalization that have been conserved during Chordate evolution.

Another example of an important regulatory gene, whose function has been remarkably well conserved during evolution, is the *Pax6* gene. *Pax 6* encodes a transcription factor that has been implicated in the development of eyes and portions of anterior nervous systems, throughout the animal kingdom. Recently the regulatory region of the *C. intestinalis Pax6* has been analyzed and compared with that of mouse and *Drosophila Pax6* (Irvine *et al.*, 2008). The results showed a similar level of complexity in cis-regulatory elements on a gross scale. The three species have regulatory elements located in a large intron near the 5' end of the gene, able to drive transcription in photoreceptors, brain and nerve cord. Both *Drosophila* and *Ciona* have major enhancers for brain and nerve cord upstream of the transcription start site. Despite these similarities in the genomic organization, no sequence similarities have been found using blast alignment, suggesting that after divergence of a common ancestry sequence similarities have been obscured by binding site turnover and rearrangements (Irvine *et al.*, 2008).

A final example derives from studies of the *Msx* family of transcription factors. Members of the *Msx* family are among the regulatory genes expressed from early to late embryonic stages and showing several different functions during development. *Msx* genes show highly conserved functions during evolution in neural patterning and dorso-ventral subdivision of the embryonic neuroectoderm (D'Alessio and Frasch, 1996; Bendall and Abate-Shen, 2000).

Only one *Msx* gene is present in *Ciona* genome and it shows an interesting expression pattern in the mesenchyme and nervous system precursors starting from the early gastrula stage (Aniello *et al.*, 1999). In larval stages, the expression is maintained in the sensory vesicle and in the visceral ganglion. Loss of function experiments, via morpholino oligonucleotide microinjection, gave some information on the role played by Msx in muscle differentiation, but they failed to give crucial information on *Msx* function in nervous system development, probably because this later function was masked by early expression (Imai *et al.*, 2006). In this case, the characterization of the regulatory region could give important insights on the regulation of this gene, in different tissues and at different developmental stages, and could help to place *Msx* gene in the network leading to anterior CNS regionalization. It has been demonstrated that the specific expression of *Msx* in the neural precursors is regulated by a 30-bp region that is able to recapitulate the endogenous expression in the nervous system both at neurula and tailbud stages. Comparison of the *Ci-msx* promoter with that of murine *Msx-1* did not show any sequence homology, suggesting that these genes are regulated by different networks. Unfortunately, although some putative binding sites have been identified in the 30-bp region, the factors responsible for *Msx* activation have not yet been identified (Russo *et al.*, 2004). Further studies are necessary to reconstruct its specific role in the nervous system regionalization and the genetic cascade in which it is involved.

It is possible to argue from the fore mentioned examples that we have only begun to exploit the enormous potential of regulatory region analyses in *Ciona*. Recently, a database has been created containing information on regulation of tunicate genes collected from literature. It includes information regarding the minimal promoter length, the transcription factors involved and their binding sites, as well as the localization of the gene expression (Sierro *et al.*, 2006). Further improvements, such as the inclusion of information on the regulation of *Halocynthia roretzi*, *C. savignyi* and *O. dioica* genes, and continuous updating will greatly contribute to reconstruct genetic networks.

CNS sensory organ differentiation

The brain vesicle of ascidian larvae contains two distinct pigmented sensory organs clearly visible through its transparent body. The more anterior pigmented sensory organ, the otolith, is involved in the perception of gravity, whereas the more posterior one, the ocellus, is involved in the perception of light stimuli. The two sensory organs are responsible for the swimming behaviour of the larva (Tsuda *et al.*, 2003). The otolith is composed of a large spherical cell attached to the ventral wall of the sensory vesicle by a narrow stalk. It contains a single pigment granule that occupies most of the cell body. The ocellus is composed of a cup-shaped pigmented cell, a number of photoreceptors and three lens cells (Dilly, 1969; Nicol and Meinertzhagen, 1991). The ascidian ocellus is considered a simple eye. The presence of photoreceptors, of 3 lens cells and of a pigmented cell in the ocellus, suggests some similarity with vertebrate eye. The ascidian “simple eye”, with its close association of the pigment cell and photoreceptors, may thus offer a “unique possibility” to study the developmental programs bringing to both melanization and photoreceptors differentiation. The “unique possibility” is related not only to the simplicity of the system but also to the very well characterized lineages of photoreceptor and pigment cells.

The molecular mechanisms regulating ascidian pigment cell development are not yet clear. The two pigment cells arise from the paired a8.25 blastomeres that are positioned bilaterally in the gastrula ascidian embryo and will give rise to the a9.49 pair at neural plate stage (Fig. 2A, B). It is known that determination of a8.25 cells as pigment cell precursors requires direct inductive influence from the nerve cord precursor cells at the gastrula stage. At this stage the a8.25 blastomeres constitute

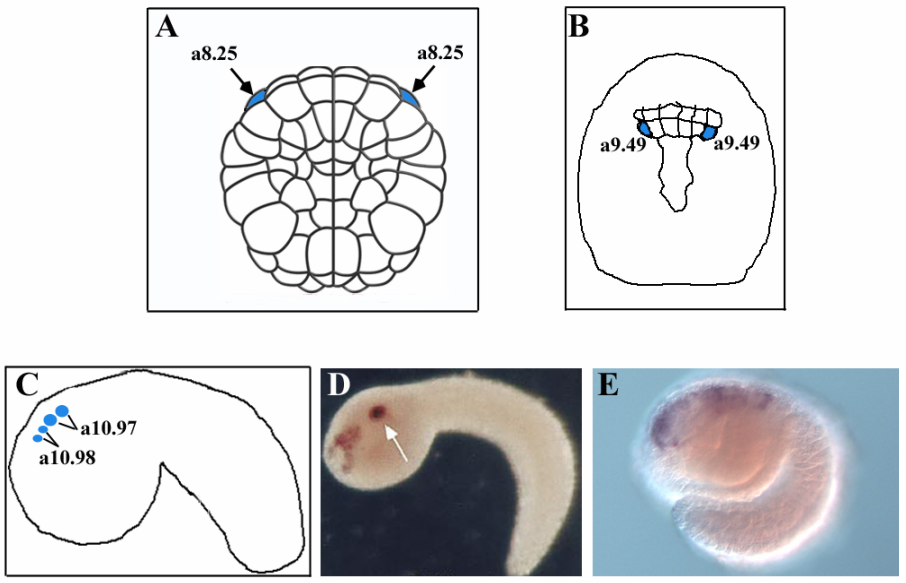

Fig. 2 Cell lineage of pigment cell precursors (light blue) in ascidian embryos at 110 cell, neural plate and tailbud stages (A-C). Expression pattern of C*hordin* gene in *Halocynthia roretzi* (D) and *Ciona intestinalis* (H) embryos at tailbud stage. White arrow in D indicates C*hordin* expressing cell in the sensory vesicle. Image D is from Darras *et al.*, 2001.

an equivalence group in the sense that both have the potential to form either an ocellus or otolith (Darras and Nishida, 2001). The choice to adopt either otolith or ocellus cell fate is made after neural tube closure at early tailbud stage and requires cell-cell interactions. As the neural tube closes, the four cells derived from the division of the two a8.25 cells, the a10.97 and a10.98 pairs, converge and intercalate along the anterior-posterior axis in order to align along the midline (Fig. 2C). The a10.98 pair gives rise to part of the brain vesicle, the anterior a10.97 forms the otolith while the posterior one differentiates into the ocellus pigment cell. In the ascidian *H. roretzi*, the choice to become ocellus or otolith seems to be influenced by the antagonistic effect of *BMP,* expressed at tailbud stage in the four tyrosinase positive cells (a10.98 and a10.97 pairs), and *CHORDIN,* secreted from the adjacent posterior cell (Fig. 2D) (Darras and Nishida, 2001). It seems that high doses of *BMP* inhibit pigmentation (as in the a10.98 pair), intermediate doses allow the development of an otolith (in the anterior a10.97) and low levels lead to ocellus development (in posterior a10.97) (Darras and Nishida, 2001). Nevertheless this mechanism seems not to be universal, as suggested by the wide expression pattern of *CHORDIN* in the anterior CNS of *C. intestinalis* tailbud embryos (Fig. 2E; personal unpublished data). This evident difference render it unlikely that in *Ciona* BMP/Chordin could be the sole responsible for the induction of the posterior a10.97 blastomere to form the ocellus. Further studies are necessary to elucidate the molecular mechanisms underling early and late regulation of pigment cell formation. These studies will take advantage of the analysis of molecular markers specific for the pigment cell precursors at different stages of development. Some genes involved in the process of melanization have been identified. Tyrosinase expression, as well as its enzyme activity, has been detected in the two types of pigment cells in several ascidian species, suggesting that this enzyme is conserved as key enzyme for melanin synthesis and that melanin has similar chemical characteristics to the melanin of vertebrates. Moreover, the expression pattern of a *Trp* (Tyrosinase related protein) has been analysed as well as a *Mitf* gene (Microphthalmia transcription factor); both show an expression pattern correlated, in some developmental stages, with restriction of pigment cell fate indicating their potential involvement in this process as in higher Chordates.

In the ascidian *H. roretzi*, as in higher Chordates (Camacho-Hubner and Beermann, 2000; Camacho-Hubner *et al.*, 2002; Camp *et al.*, 2003; Goding, 2000), Tyrosinase Gene Family (TyGF) has been used as a model to approach studies on "pigmentation programs" (Kusakabe *et al.*, 2001). Firstly it has been identified a *HrTyr* promoter fragment able to direct lineage-specific and developmentally correct expression of *HrTyr* during embryogenesis. A search for conserved recognition sequences in this promoter has revealed the presence of several putative Pax3-binding consensus sites; the data that over-expression of *HrPax*-3/7 is able to induce ectopic expression of *HrTyr* (*Halocynthia roretzi Tyrosinase)* have reinforced the hypothesis of a direct relation between *HrPax*-3/7 and *HrTyr* (Toyoda *et al.*, 2000). A parallel approach on the *HrTRP* (*Halocinthia roretzi* Thyrosinase Related Protein) promoter has permitted the identification of two Otx binding consensus sites involved in *HrTRP* expression; here too, Hroth overexpression can transactivate this promoter in an Otx site-dependent manner,

confirming a direct function exerted by Hroth on *HrTRP* expression (Dehal *et al.*, 2002). The mechanisms of Tyrosinase/TRP activation seem therefore to be conserved on most aspects during chordate evolution (for a review see Murisier and Beermann, 2006). In higher Chordates, members of the Microphthalmia (Mitf) transcription factor family play a central role in specification of pigment cell lineage (Aksan and Goding, 1998; Hotta *et al.*, 2000). The absence of Mitf binding sites in *HrTyr* and *HrTRP* minimal promoter elements, compared with higher Chordates, is a little bit surprising, given that molecular data from *H. roretzi* strongly supports the antiquity of the association of the Mitf family members with pigment cells (Yajima *et al.*, 2003). One possibility is that Mitf, at least in *H. roretzi*, is not involved in the regulation of *HrTyr* and *HrTRP* expression; or that *HrMitf* boxes, located in the far upstream region, are not necessary but can contribute to a full efficient expression of Tyrosinase(s) during *Halocynthia* embryogenesis; or that HrMitf/*HrTyr-HrTRP* interaction is not direct but mediated by other factors.

The scenario emerging from the data collected so far in ascidians point to an evolutionary conservation of most factors involved in pigment cells differentiation. What about photoreceptors? The question is very intriguing given that photoreceptor and pigment cell precursors, in the ascidian *C. intestinalis*, share at the late gastrula stage common factors, as Mitf and BMP5/7, that slightly later, at the neurula stage, become localized specifically in pigment cell precursors (data not shown). Studies on photoreceptor differentiation in ascidians have produced very few data so far.

Opsin (*Ci-opsin1*) (Kusakabe *et al*., 2001) and arrestin (*Ci-arr*) (Nakagawa *et al.*, 2002) genes have been isolated and characterized as photoreceptor-specific markers from the ascidian *C. intestinalis*; a 3kb *Ci-arr* promoter region has been demonstrated to recapitulate the expression of the endogenous gene (Ikuta *et al.*, 2004). These genes are directly involved in the visual cycle; studies on their transcriptional regulation could therefore help to clarify the mechanisms involved in terminal differentiation of photoreceptors.

Only recently a key factor required for the differentiation of vertebrate eye, the *Rx* gene, has been cloned from *Ciona* (*Ci-Rx*). "Loss of function" experiments have indicated that it is required for ocellus development. In particular, larvae lacking Rx function do not develop ocellus pigment cell, lack photoreceptors and are unable to response to light stimuli (D'Aniello *et al.*, 2006). Furthermore, a *Ci-Rx* regulatory region, that recapitulate the expression of the endogenous gene, has been identified (D'Aniello *et al.*, 2006). This *Ci-Rx* "eye enhancer" could provide interesting keys to unravel the genetic circuits controlling a step just prior to the terminal differentiation of photoreceptors. Studies on the transcriptional regulation of genes as *BMP* and *Mitf* in ascidians could finally be instrumental to shed light on the mechanisms/factors involved in the initial choice pigment cell-photoreceptor.

Recent work has suggested that pigmented cells of the ocellus and otolith are necessary for sensing light and gravity, respectively (Sakurai *et al.*, 2004; Tsuda *et al.*, 2003). The behaviour of ascidian larva has long been studied illustrating that ascidian larvae present phototactic and geotactic responses. The larvae become sensitive to light about 4 hours after hatching, responding to decreased light intensity by swimming more actively. A direct proof for the role of pigmentation in ascidian larval physiology have been obtained only recently, taking advantage of two mutant lines of *C. savignyi* that are unable to make melanin and thus lack pigment in the larval sensory structures. Behavioural studies on non pigmented offspring demonstrated that unpigmented larvae are unable to detect source of light and consequently are unable to seek out the shaded location preferred by their wild type siblings (Jiang *et al.*, 2005). Moreover, the *C. savignyi* mutant larvae lacking pigmentation do not behave properly in response to gravity. Other information could come from the physiological studies of other naturally occurring mutants of *C. intestinalis* isolated in the Gulf of Naples, presenting several defects in pigmentation ranging from total absence of pigmented cells to presence of only one pigmented cell (otolith or ocellus) (Sordino *et al.*, 2008).

Conclusions

The new molecular tools and the great progresses in deciphering ascidian genome and chromosomal mapping, allowed moving to a further and more advanced step in the comprehension of the complex chordate body organization. From the study of single genes or single genetic cascades, it is now possible to integrate and interconnect the regulatory networks that underlie the organization, function and development of the ascidian nervous system. These progresses refer overall to early steps of neural induction but represent the milestone for future reconstruction of late CNS regionalization and differentiation.

Despite the obvious morphological differences and the divergence of regulatory sequences that, in some cases, have been observed, it seems that most of the main CNS developmental programs are conserved between ascidians and vertebrates. This result strongly spurs to use ascidians, with their compact genome and relatively simple CNS, as model system to characterize some of the conserved and fundamental steps of chordate neurogenesis. To this aim, it will be very important in the next years to integrate the functional studies (eg. morpholino loss of function) of genes involved in a specific patterning with the characterization of the corresponding regulatory elements.

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