From the American to the European amphioxus: towards experimental Evo-Devo at the origin of chordates

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ABSTRACT Pallid anchovy fillet, friendly filtering, peacefully laying and little lancelet are some of the nicknames and adjectives the cephalochordate amphioxus has received throughout the last two centuries. Traditionally regarded as the living representative of the last ancestor of vertebrates, amphioxus has recently been promoted to the privileged position of being the most ancient chordate. The preliminary analysis of its prototypical genome is nearly completed, and its hidden secrets towards the understanding of the primitive chordate and deuterostome genomes will soon see the light. Amphioxus embryonic development and body plan have remained in evolutionary stasis since the cephalochordate lineage split from the chordate ancestor about 500 million years ago. In contrast, amphioxus research is far from being at a standstill; in Europe, thanks to the international cooperation and the Banyuls Oceanographic Station, amphioxus embryos are obtained on demand during the spawning season. We summarise here our progress towards the dream of the experimental manipulation of the amphioxus embryo, to enter the era of Experimental Evo-Devo.

KEY WORDS: amphioxus, Evo-Devo, chordate, gene duplication, vertebrate

Commonality of embryonic structure reveals community of descent. As simple as it sounds today, the striking sentence of Charles Darwin (1859) highlighting the importance of embryonic development to indicate evolutionary relationships was not seriously taken into account by geneticists and evolutionary biologists until late 20th century, when molecular genetics showed that most gene networks responsible for embryonic development and functioning of metazoans were conserved; evolution may have well worked by tinkering and bricolage of a basic genetic toolkit to shape animal body plans and adaptations.

In Comparative Zoology, Evolution and modern Evolutionary Developmental Biology (Evo-Devo), the origin of vertebrates has always received much attention, most probably due to a vertebra-and anthropo-centrist view of scientists (Duboule, 2007), but also due to the fact that the origin of vertebrates involved the appearance of several intriguing innovations. Among those, vertebrae, regionalised anterior brain, neural crest cells and paired limbs evolved in the ancestor of vertebrates and generated, among other complex vertebrates, ourselves. The nature of this ancestor has been subject of debate, and the position of the clades accompanying vertebrates in the phylum chordates was, until very recently, controversial.

Lancelets, or amphioxus, are small marine animals with a fish-like shape that spend most of their life partially burrowed in the sand, filtering sea water trough their jawless mouth, in search of their main food, unicellular algae. Currently, the subphylum Cephalochordata comprises 29 species of amphioxus (Pos and Boschung, 1996). The brilliant embryologist Alexander Kowalevsky (1867) already noted that amphioxus shared key anatomical features with vertebrates, such as a hollow dorsal nerve tube, an endostyle, a segmented body derived from somites and a post-anal tail (Fig. 1). However, amphioxus lacks some vertebrate key characteristics, including migratory neural crest cells, an endoskeleton, a highly regionalised brain, or paired sense organs (Shimeld and Holland, 2000). Fossil chordates with similar morphology to modern amphioxus have been described (Chen et al., 1999),

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indicating that amphioxus represents, morphologically, a close living proxy of an early chordate. Recent molecular data indicate that not only its body plan but also its genome is the best representative of the ancestral chordate genome, and perhaps one of the best approaches so far to the ancestral «complex Urbilateria» (Baguñà and Riutort, 2004).

The changing (and definitive?) phylogenetic position of amphioxus: implications for the ancestral chordate and the invertebrate/vertebrate transition

Although amphioxus was originally described as a mollusc (Pallas, 1774), during the late 19th century it became clear that either cephalochordates or urochordates (ascidians) were the most basal chordates (Kowalevsky, 1867). The prevalent view on the origin of chordates during the 20th century was that urochordates represented the most basal branch of chordates (Fig. 2A). In this scenario, the chordate (and deuterostome) ancestor was probably a sessile animal similar to the ascidian adult that would have given rise to the ancestor of amphioxus and vertebrates by neoteny (Sly et al., 2003). However, in the last few years, the position of cephalochordates and tunicates has been reversed. By analysing a large amount of molecular data, Delsuc and colleagues (Delsuc et al., 2006) found that cephalochordates branched earlier than it was thought, representing the sister group of a urochordate–vertebrate clade (Fig. 2B). This change in the chordate phylogeny has profound implications for the understanding of the evolution of body plans. First, it suggests that the ancestral chordate (and perhaps the ancestral deuterostome) was a free-living, worm or fish-like shaped organism, with a series of pharyngeal gill slits. Second, it implies that the origin of vertebrates may not occurred by neoteny of a urochordate-like sessile animal. Finally, it indicates that urochordates and echinoderms have a derived body plan, whereas amphioxus and vertebrates may have retained body plans more comparable to the ancestral deuterostome.

That amphioxus is the best representative of the first chordate has insightful implications, which help to clarify the evolutionary pathways within chordates and to vertebrates. Two examples are the origin of the Spemann-Mangold organizer, and the origin of the neural crest. The discovery of the former (Spemann and Mangold, 1924), a small group of cells in the early gastrula with inductive properties that establish the vertebrate body plan, was a landmark in experimental embryology. However, the evolutionary origin of the organizer itself was obscured until the reversal of the positioning of cephalochordates and urochordates, and the publication of recent molecular data. First, although the ascidian larva is vertebrate-like in shape, its embryonic development is deployed neither with the presence of an organizer-like territory, nor by large inductive mechanisms (Kourakis and Smith, 2005). Second, pioneering work in experimental embryology in China in the 1960s suggested that the dorsal part of the amphioxus blastopore is homologous to the vertebrate organizer, having similar inductive properties (Tung et al., 1962). Finally, Yu and colleagues recently reported that, at the molecular level, the amphioxus gastrula may have such an inductive territory with separate signaling centers to pattern the Antero-Posterior and Dorso-Ventral embryonic axis very similar to vertebrates (Yu et al., 2007). Altogether, these data clarify the origin of the organizer: it appeared, at least, in the chordate ancestor, and therefore is not a vertebrate novelty, but it was secondarily lost in urochordates, maybe as an adaptation to their small size and rapid and regulative development (Garcia-Fernàndez et al., 2007).

Neural crest cells have a major impact in vertebrate development. They differentiate into a vast array of cell types, generating a wide variety of tissues and organs, such as craniofacial skeleton, spinal ganglia, parasympathetic and sympathetic nervous systems and teeth (Hall, 1999; Trainor et al., 2003). Although most components of the gene networks involved in neural crest specification are expressed in the border of the amphioxus neural tube, in a similar pattern to vertebrates, no cell migration has been reported in amphioxus at these embryonic stages and locations (Barrallo-Gimeno and Nieto, 2006; Meulemans and Bronner-Fraser, 2004). Intriguingly, a population of cells in the amphioxus neurula shows migratory capabilities and may have the ability to perform epithelial/mesenchymal transition (Benito-Gutiérrez et al., 2005), but they are not located near the dorsal neural tube. On the other
hand, a cell population in the ascidian urochordate *Ecteinascidia turbinata* behaves, at least partially, as canonical neural crest cells (Jeffery *et al.*, 2004). Even if all these data are taken with caution, the evolutionary path to the origin of genuine neural crest cells seems clearer: between cephalochordates and vertebrates, some cells adjacent to the neural tube acquired migratory capabilities, and *bona fide* neural crest cells evolved later, along the lineage leading to vertebrates.

**A genome to dream of...**

A statement in a classic Biology textbook from the 1960s well represents the importance of the lancelet in understanding vertebrate origins: “If amphioxus had not been discovered, it would have to have been invented” (Grove and Newell, 1961). The authors refer to the prototypical condition of the amphioxus embryo and body plan; they are as one would have expected of the ancestor of vertebrates.

Molecular genetics in amphioxus started in 1992, with the cloning of the first homeobox gene, *AmphiHox3*, equally related to the three human Hox3 genes (Holland *et al.*, 1992). This early finding was partly in agreement with the early ideas of Susumu Ohno, who anticipated that during vertebrate evolution, one or several genome duplications took place (Ohno, 1970). Ohno nicely linked those genome duplication events to the concept of neo-functionalisation of duplicated genes that would be the raw material for further innovations without the constraints of strong purifying selection. Although gene co-option through changes in *cis*-regulatory sequences has overtaken gene duplication as the main player in our understanding of evolution (Carroll, 2005), the main question of whether vertebrates are octoploids or not has been on hot debate in many forums (e.g. (Furlong and Holland, 2002)). The full sequencing of the human genome prompted their authors to propose that the amphioxus genome would give the answer to such a question (Consortium, 2001). After the analyses of the urochordate *Ciona intestinalis* genome (Dehal *et al.*, 2002) the first global analysis of the amphioxus genome will soon unravel the details of Ohno’s hypotheses.

Nonetheless, previous analyses of individual genes, gene networks and gene clusters have already indicated that, as a general rule, amphioxus has single members for nearly all vertebrate gene families (Holland *et al.*, 2004). Paradigmatic examples are the Hox cluster (Garcia-Fernández, 2005b) and its evolutionary sister, the ParaHox cluster (Brooke *et al.*, 1998). Mammals have four Hox clusters with a representation of 13 paralogous groups. None of these clusters has more than 11 of these paralogous groups, whereas amphioxus has a single Hox cluster, with one member of each paralogous group, in the single, and at the same time, richest, Hox cluster so far described. Moreover, amphioxus contains a 14th Hox gene, *AmphiHox14* (Ferrier *et al.*, 2000). This finding was initially puzzling, until a 14th Hox gene was found in coelacanth and shark (Powers and Amemiya, 2004):

*AmphiHox14* did not represent an amphioxus oddity; instead, Hox14 was lost in the lineage to tetrapods (Garcia-Fernández, 2005a). Intriguingly, amphioxus also possesses a *Hox 15* gene (Holland *et al.*, 2008).

Interestingly, the expression of Hox genes in amphioxus also exemplifies the use of amphioxus developmental genes as tracers of hidden homology. The amphioxus neural tube has no evident morphological segmented boundaries, like rhombomeres. However, Hox genes are expressed in a staggered collinear manner, with nested expression (Fig. 3). This is reminiscent to the rhombomere nested phase in which the same paralogous Hox genes are expressed in the vertebrate hindbrain. Although inferring homology through gene expression comparison may not reflect always true morphological homology, these similarities strongly suggest that a large region of the amphioxus nerve cord is equivalent to the vertebrate rhombencephalon, and that the highly developed forebrain of vertebrates evolved from a small region of the anterior neural tube of the chordate ancestor, which in amphioxus is represented by the frontal cerebral vesicle (Holland and Takahashi, 2005; Lacalli, 2006).

**…but also a modern genome**

Although the amphioxus genome seems close to the prototypical chordate genome, it is not a genome that froze in an ancestral state, without change. Amphioxus may be in morphological stasis...
and its genome might have not experienced high overall evolutionary turnovers, like in other derived lineages (Consortium, 1998; Dehal et al., 2002). Nevertheless, after 500 Million Years (MYs) of lineage-specific evolution from the ancestor of chordates, some amphioxus-specific footprints are present, even though they have not distorted the morphology or the basic set of genes and gene networks.

We found one of these footprints while studying somitogenesis, analysing whether or not the cycling mechanism operating in vertebrates is ancestral to chordates (Pourquié, 2003). Somites are an evolutionary innovation that appeared at the origin of chordates. Amphioxus possesses epithelial axial somites without differentiation into sclerotome and dermamyotome (Pourquié, 2001). Unexpectedly, not one but eight basic helix-loop-helix genes of the hairy family were found (Minguillón et al., 2003). The expression patterns of four of those genes in amphioxus were striking: they were expressed in non-overlapping domains in the embryo, including somites, nerve cord and gut (Fig. 4B). A careful analysis of these expression patterns showed that the summation of all the patterns represents the expression of the single mouse hairy gene. These results suggest that AmphHairy genes have undergone a process of sub-functionalisation due to gene duplication, in agreement with the DDC model (duplication/degeneration/complementation) proposed by Force and colleagues (Force et al., 1999).

According to this model, after gene duplication of a complex regulatory region, some of the enhancers are differentially maintained or lost among the newly created duplicates. Therefore, in order to ensure the complex, multiple expression pattern of the ancestral, single gene, none of the duplicated copies can be lost by non-functionalisation (degeneration). Developmental genes, which often have very complex regulatory regions, are more prone to undergo sub-functionalisation. This may well be the reason why they have had a much lower rate of loss in vertebrate genomes, after polyploidisation, than housekeeping or constitutive genes. We further investigated the sub-functionalisation process of the amphioxus hairy genes, by analysing the 5’ regulatory regions of AmphHairy A-D (Jiménez-Delgado et al., 2006). As expected, several conserved regions were detected among some duplicates (Fig. 4A).

Hence, although amphioxus shows high constraints in its genome, it is a contemporary genome, with remnants of particular duplications and expansions. The complete analysis of the lancelet genome will bring to light other cases apart from those already reported (Minguillón et al., 2002); this analysis may also help to understand genome dynamics in an almost unique case in which everything necessary to conserve the basic chordate body plan has been retained; as examples, consider the recurrent clustering of Iroquois genes (Irimia et al., 2008) or the expansion of the tyrosine kinase family (D’Aniello et al., 2008).

**The embryo: from Tampa Bay to Banyuls sur mer**

It is a classic recipe: a genomic and developmental animal model system must have a model genome (as amphioxus has), a model embryo (which it also has), and these embryos should be easy to obtain. Until recently, the friendly lancelet did not fit this recipe. The first experimental studies on amphioxus embryology, during the 19th and 20th centuries, were carried out on embryos caught in the wild and under difficult conditions. Three main species are currently being studied: the American, Branchiostoma floridae, found in the shallow waters of Tampa Bay (Holland and Yu, 2004), the Asian, B. belcheri (Zhang et al., 2007), and the European, B. lanceolatum (Fuentes et al., 2004). Most developmental genetics studies have been performed on *B. floridae*, and the genome sequenced is also that from *B. floridae*. The study of embryos of *B. floridae* has been possible in recent years thanks to the hard work of Nick and Linda Holland (Scripps Institution of Oceanography, San Diego, USA). In July-August every year, up to a dozen amphioxus researchers were kindly hosted by Nick, Linda, and John Lawrence, from the University of South Florida. The main aim was to obtain *B. floridae* embryos. And this was not trivial. Populations of *B. floridae*, as most species of amphioxus, spawn synchronously in the wild, at sunset of very few days a year (Holland and Yu, 2004; Stokes and Holland, 1996). This means that experimentation with live embryos is a challenge.

![Fig. 4. Schematic representation of correlation between putative conserved sequence elements and Hairy expression territory. (A) Schematic representation of the 5' region of amphioxus HairyA, B, C and D genes showing conserved non-coding regions. Five different conserved elements were identified and are shown in different colours. Box2 (blue) would correlate with the expression of HairyB, HairyC, HairyD in somites (s) and presomitic mesoderm (psm). Box3 (green) with HairyC and HairyD in notochord (n). Box4 (yellow) with the expression of HairyA, HairyC, and HairyD in gut endoderm. Box5 (pink) with the expression of all four genes in neural tube (nt). Positions of the anterior (A)-posterior (P) and dorso (D)-ventral (V) axes are indicated. (B) Schematic representation of a lateral view of an amphioxus neurula showing the composite expression of AmphHairy A to D. Antero (A)-posterior (P) and dorso (D)-ventral (V) axes are indicated.](image)
embryos was a limiting factor for experimental research. As such, little literature includes gene or embryo manipulation (Beaster-Jones et al., 2007; Benito-Gutiérrez et al., 2005; Schubert et al., 2005).

Hatschek, Conklin or Kowalevsky, among other classic embryologists, studied the European species, *B. lanceolatum*, (Conklin, 1932; Hatschek, 1881; Kowalevsky, 1867) obtained in several research marine stations around Europe. But European molecular geneticists interested in the invertebrate/vertebrate transition, either concentrated in echinoderms or urochordates, much more amenable to experimentation, or the few in love with amphioxus followed the path to the Florida lancelet every summer. Few years ago, however, a European initiative, led by French groups, and including Spanish, Italian and German labs, started an ambitious collaboration to study the natural reproduction of *B. lanceolatum* in the beaches near the Laboratory Arago, Banyuls-Sur-Mer, France. This initiative ended with major success: we were not only able to obtain embryos in the lab coincidently to the natural spawning nights in the field (April to June), but also developed a technique to make the animals spawn in the lab independently of field conditions (Fuentes et al., 2007; Fuentes et al., 2004). In practice, this means that amphioxus embryos can be obtained in Banyuls on demand, during the spawning season. And this, of course, opens the door to develop amphioxus as a fully experimental model system. Here we present an illustrated development table of *B. lanceolatum*, from fertilization to larval stages (Fig. 5 A-H), and scanning microscope pictures of gastrula stage (Fig. 5I).

The available amphioxus genome is that of *B. floridae*, whereas it is now easier to obtain *B. lanceolatum* embryos, and *B. belcheri* research is growing in China and Japan (Wang et al., 2005; Zhang et al., 2007). A list of genomic and experimental tools of each species is shown in Table 1. Thus not only one, but three species of amphioxus will serve, in addition, to reveal, towards phylogenetic footprinting, conserved gene regulatory regions to show up those functional elements in the grounds of modern comparative and functional genomes.

The Barcelona lab: genome and embryos *a la carte*

The amphioxus research in Barcelona started when JGF, inspired by his post-doctoral experience with Peter Holland in Oxford (Garcia-Fernàndez and Holland, 1994; Holland et al., 1994), was lucky to be joined by a group of scientists whose main interest was Evo-Devo at the chordate/vertebrate origin, using amphioxus as a model organism. We initially mainly

| TABLE 1 | GENOMIC RESOURCES AND EMBRYO AVAILABILITY FOR THE THREE MAINLY USED BRANCHIOSTOMA SPECIES |
|---|---|---|
| **B. lanceolatum** | **B. floridae** | **B. belcheri** |
| Whole genome sequence | Joint Genome Institute [http://genome.jgi-psf.org/Brafl1/Brafl1.home.html] | S38 in Genbank, Wang et al. 2005 |
| ESTs | in process | From egg, gastrula, neurula, larva and adult [http://dolphin.lab.nig.ac.jp/bball.html] |
| Phage Genomic DNA library | Cañestro et al., 2000 | From adult gut (Luan et al., 2007), notochord (Suzuki and Satoh, 2000), neural embryonic tissue (Dong et al., 2005), neurula [Mou et al., 2002, Lin et al., 2004] |
| cDNA library | From posterior part of adult (Sato et al., 2003) | |
| Embryos availability | Multiple spawnings; synchronous or not during the spawning season (from April to June); Heat-schock every day during the spawning season; Day/night cycle controlled | Multiple synchronous spawnings with 1-2 weeks intervals during the spawning season; Electric pulse the days of spawning in the field | Few contigous days per year |
| Natural spawning | | |
| Induced spawning | | No artificial induction |
focused on amphioxus homeobox genes (Brooke et al., 1998; Ferrier et al., 2000; Minguillón and Garcia-Fernàndez, 2002) and the data obtained in our laboratory served to suggest global hypothesis on the evolution of homeobox clusters in metazoans (Garcia-Fernàndez, 2005a; Garcia-Fernàndez, 2005b; Minguillón and Garcia-Fernàndez, 2003). Later we expanded as well the research to more global studies: the evolution of nervous system and mesodermal derivatives, genome evolution, alternative splicing and phylogenies are among interests in the recent years (e.g. Benito-Gutiérrez et al., 2006; Irimia et al., 2007a; Irimia et al., 2007b; Irimia et al., 2007c; Irimia et al., 2007d).

As an example, recent work on the free amino acid composition of the *B. lanceolatum* nerve cord has shown that amphioxus contains intermediate amounts of these amino acids compared to invertebrates and vertebrates. This represents a new and promising research line that could give insights into the evolution of neurotransmission, since some of them, such as D-Aspartic acid (D-Asp, the D form of L-Aspartate) and NMDA (the methylated form of D-Asp) among others, are implicated in neuronal and hormonal activity (D’Aniello et al., 2007; D’Aniello and Garcia-Fernàndez, 2007; Pascual-Anaya and D’Aniello, 2006).

Notwithstanding, the main goal of our team is to develop the amphioxus as an ambitious model system for Experimental Evo-Devo. For instance, we managed to establish a long-term culture of the *B. lanceolatum* nerve cord has shown that amphioxus contains intermediate amounts of these amino acids compared to invertebrates and vertebrates. This represents a new and promising research line that could give insights into the evolution of neurotransmission, since some of them, such as D-Aspartic acid (D-Asp, the D form of L-Aspartate) and NMDA (the methylated form of D-Asp) among others, are implicated in neuronal and hormonal activity (D’Aniello et al., 2007; D’Aniello and Garcia-Fernàndez, 2007; Pascual-Anaya and D’Aniello, 2006).

As a first step, we managed to establish a long-term culture facility for amphioxus in a “dry lab” (not a marine station). To our knowledge, Barcelona hosts the only dry lab where amphioxus are maintained, and spawning is induced under controlled conditions. Spawning is induced not only the day but also at the time that the researcher wishes: no longer at the sunset, with long nights of work ahead, but at noon, after succeeding in inverting the day/night cycle of the amphioxus cultures (Fuentes et al., 2007).

**The wishes, the prospects, the future**

We anticipate that amphioxus research will grow and give interesting new surprises to developmental and evolutionary biologists. The research community is increasing and the imminent publication of a draft of the amphioxus genome may be a breakthrough. The friendly little anchovy fillet, peacefully lying on the sand, may become nervous in our fish tanks, at the doors of the experimental genetics era, thanks to the collaborative efforts of researchers world-wide. A country song by the marine biologist Philip Pope concerning amphioxus became a favourite at the summer courses of Cold Spring Harbour Laboratories; the chorus can be found elsewhere (Garcia-Fernàndez, 2006): *It’s a long way from Amphioxus. It is a long way to us.* Experimental Evo-Devo in amphioxus is a long way, it is a long way for us, but an amazingly exciting one. Having amphioxus eggs injected and developed, is a nice milestone on the way. Here we show, for the first time and to set up manipulation systems in this species, fluorescent *B. lanceolatum* embryos (Fig. 6), born on demand and injected in our resources, the AmphíBCN lab.

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