19 Cephalochordates

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CONTENTS

19.1 HISTORY OF THE MODEL

Amphioxus are small, worm-like animals that resemble a fish without a head or a skeleton. They live burrowed in the sand of temperate and tropical costal areas, usually at shallow depths (1–50 m). Amphioxus, also called lancelets, is the common name for members of the cephalochordate clade. The first description of amphioxus came from a Chinese legend: Wenchang (or Wen Chang), the literature deity, was traveling around the world in search of new knowledge on the back of his pet crocodile. When the crocodile died in the Bay of Xiamen, larva emerged from its corpse. These "larva" were amphioxus, and even today the Chinese call amphioxus "Fish of the God of Literature" or "Wenchang fish" (Stokes and Holland 1998; Feng et al. 2016; Holland and Holland 2017). These animals are consumed as food in some Chinese regions, although the amphioxus population greatly decreased in the Bay of Xiamen during the second half of the 20th century.

While much more abundant in China than in Europe, the first scientific description of a cephalochordate came from the German zoologist and botanist Peter Simon Pallas in 1774, who named it *Limax lanceolatus* (Pallas 1774). He could only observe two fixed adult specimens from the Cornwall coast, UK, and classified amphioxus as a mollusk. In 1834, Gabriele Costa, a zoologist in Naples, Italy, described amphioxus as a fish and hypothesized it could represent the "missing link" between invertebrates and vertebrates (Costa 1834). He was able to observe live animals and described the oral cirri around the mouth as gills. For this reason, he gave the name *Branchiostoma* to the genus ("branchio" for "gills" and "stoma" for "mouth"). In 1836, William Yarrell, who was unfamiliar with Costa's work but knew about the description by Pallas, proposed "lancelet" as a common name for specimens from the Cornwall coast and changed the genus name *Limax*, given by Pallas, to *Amphioxus* ("amphi" for "both sides" and "oxus" for "pointed") (Yarrell 1836). Later on, the genus name became *Branchiostoma*. However, Yarrell is at the origin of the two common names of cephalochordate animals: amphioxus and lancelet. Thereafter, many zoologists developed an interest in amphioxus because of its proposed key evolutionary position as a close relative of vertebrates and made in-depth descriptions of its morphology; however, these zoologists were only working with adult specimens. The first researcher who described amphioxus embryos was the Russian embryologist Alexander Onufrievich Kowalevsky. After his studies
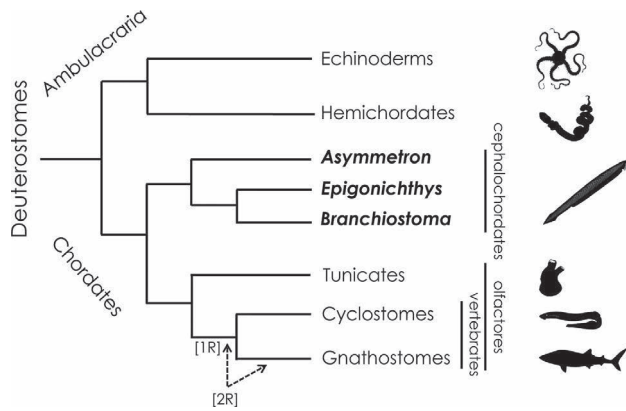
FIGURE 19.1 Deuterostome group classification. Deuterostomes are subdivided into Ambulacraria, composed of echinoderms and hemichordates, and chordates, which include cephalochordates and olfactores (tunicates and vertebrates). The three cephalochordate genera are represented in bold. The two whole genome duplications that occurred during vertebrate evolution are also indicated. The first took place before the divergence between gnathostomes (jawed vertebrates) and cyclostomes (lampreys and hagfish), whereas the position of the second is still debated.

in Russia and at the University of Heidelberg, Germany, he came to Naples in 1863 and 1864 in an attempt to obtain amphioxus embryos from local specimens (Davydoff 1960). Kowalevsky frequently collected amphioxus and kept them in his aquarium for months in hopes of the animals spawning. Finally, in May 1864, some adult animals spawned, and Kowalevsky was able, for the first time, to observe the development of amphioxus embryos (Kovalevskij 1867). He noticed that the blastula would flatten on one side that subsequently invaginated to create two embryonic layers through a process of gastrulation. His work was assembled in a manuscript thanks to which he obtained his Magister degree in St. Petersburg, Russia.

Many other zoologists became interested in amphioxus (Gans 1996), among whom were the famous Berthold Hatschek (Hatschek and Tuckey 1893) and Edwin Grant Conklin (Conklin 1932), who made many descriptions of amphioxus embryogenesis, as well as the German naturalist Ernst Haeckel, who wrote in the fifth edition of the book *The Evolution of Man*: "We begin with the lancelet—after man the most important and interesting of all animals. Man is at the highest summit, the lancelet at the lowest root, of the vertebrate stem" (Haeckel et al. 1905). However, being extant animals, cephalochordates cannot be at the root of vertebrates, but evolutionarily they are closely related; cephalochordates, together with vertebrates and their sister group the tunicates, form the chordate clade (Figure 19.1). This evolutionary proximity is one of the reasons many researchers use amphioxus as a model in research.

Therefore, the study of amphioxus development and its comparison with tunicate and vertebrate embryogenesis allows us to define ancestral traits of chordates and to understand the appearance of vertebrate-specific morphological characters.

During the second half of the 20th century, research on amphioxus slowed down in Europe and the United States while flourishing in China with the species *Branchiostoma belcheri* (Light 1923). Among Chinese researchers, Ti Chou Tung elegantly studied embryonic cell fate in amphioxus using vital staining and delicate micro-manipulations, providing the scientific community with important insights into cephalochordate development (Tung et al. 1958, 1960, 1962, 1965). Later, amphioxus entered the molecular biology era thanks to American researchers Dr. Linda and Prof. Nicholas Holland from the University of California, San Diego. They began to collect adults from the species *Branchiostoma floridae* in Tampa, Florida, during the summer of 1988 and were able to obtain embryos from *in vitro* fertilization and using gametes obtained by spawning induction of the adults through electric stimulation (Holland and Holland 1989). In collaboration with Prof. Peter Holland from Oxford University, they developed a protocol to analyze embryonic gene expression through whole mount *in situ* hybridization experiments, allowing the scientific community to renew its interest in amphioxus as a modern model to study the evolution of developmental mechanisms (Holland et al. 1992).

At the beginning of the 21st century, the development of new sequencing techniques accompanied the transition to whole-genome level studies for many organisms, including amphioxus. The first whole-genome sequence was obtained for the American species *B. floridae* (Putnam et al. 2008), followed by the genome of *B. belcheri* (Huang et al. 2012) and the genome and epigenome of the European species *B. lanceolatum* (Marletaz et al. 2018). These advances have made amphioxus a good model not only to understand morphological evolution in the chordate clade through developmental biology approaches but also to study the evolution of genome structure and function. Before any cephalochordate genome was published, multigene phylogenetic studies taking advantage of the whole genome sequencing of the tunicate *Oikopleura dioica* showed that, contrary to what was globally accepted in the community, tunicates, and not cephalochordates, are the sister group of vertebrates, with which they form the *Olfactores* clade (Delsuc et al. 2006). Comparing vertebrates and amphioxus thus gives us information on the chordate ancestor that probably had characters more closely related to those of vertebrates than previously thought!

19.2 GEOGRAPHICAL LOCATION

Cephalochordates include three genera—*Branchiostoma*, *Epigonichtys* and *Asymmetron*—with around 30–40 species described to date (Poss and Boschung 1996). All animals of this chordate group are very similar morphologically, the only major difference being that adults of the *Branchiostoma* genus species have two rows of gonads on both sides of the body, whereas *Asymmetron* and *Epigonichtys* species have only one row of gonads on the right side. Amphioxus live in the sand of the seafloor with the anterior part of their body sticking out of the sediment and feed by filtering the seawater. Cephalochordates are widely distributed, with species

described along tropical and temperate coasts in sandy sediments all around the world (Poss and Boschung 1996). The precise distribution of each species is hard to define, as historically the identification of species was only based on morphological and meristic data, which, as stated before, are not sufficiently discriminant due to the high morphological resemblance among cephalochordates. Development of molecular identification is rising and recently allowed several research groups to suggest the existence of more species than previously described (Nishikawa 2004; Nohara et al. 2005; Kon et al. 2006; Kon et al. 2007; Igawa et al. 2017; Subirana et al. 2020). Moreover, regarding Asian species, recent studies showed that western Pacific lancelet populations that were for a long period recognized as belonging to one species, *B. belcheri*, belong instead to two distinct species, *B. belcheri* and *B. japonicum* (Zhang et al. 2006; Li et al. 2013). Molecular phylogenetic data also allowed the clarification of evolutionary relationships between species and showed that *Branchiostoma* and *Epigonichtys* are more closely related to each other than to the *Asymmetron* genus (Igawa et al. 2017). Interestingly, although *Asymmetron* and *Branchiostoma* diverged between 46 and 150 Mya (Igawa et al. 2017; Subirana et al. 2020), viable hybrid embryos from *A. lucayanum* and *B. floridae* can be obtained by *in vitro* fertilization (Holland et al. 2015).

19.3 LIFE CYCLE

19.3.1 Animals in the Field

Amphioxus are gonochoric animals presenting a typical bentho-pelagic life cycle. Males and females live burrowed in the sand, and during the breeding season, they swim into the water column just after sunset and release all their gametes into the environment: hundreds of oocytes are spawned by each female, whereas males release sperm full of spermatozoids. After external fertilization, the embryo continues its development protected by the fertilization envelope, also called the chorion. Hatching occurs at the end of the gastrulation process, and the ciliated embryo continues developing to form a planktonic larva that moves thanks to both the epidermal cilia and the newly formed trunk striated muscles. The larva then metamorphoses and becomes a juvenile that returns to a life in the sediment and reaches adulthood after sexual maturation (Stokes and Holland 1998).

The duration and timing of the breeding season depend on the species, as well as the speed of embryonic and post-embryonic development. In the *B. floridae* population of Tampa Bay, the breeding season starts in early May and ends at the beginning of September (Stokes and Holland 1996). During this period, animals might spawn several times and produce new gametes more or less every two weeks. In the Mediterranean *B. lanceolatum* population of Argelès-sur-Mer, France, the breeding season starts in May and ends in July, with animals capable of spawning at least twice during this period, although, contrary to observations made for *B. floridae*, animals from the same location do not always spawn

synchronously (Fuentes et al. 2004; Fuentes et al. 2007). The two Asian species *B. belcheri* and *B. japonicum* can also spawn at least twice in the field during their reproductive seasons, which range from May to the end of July and from late April to late August, respectively (Zhang et al. 2007; Li et al. 2013). Finally, the *A. lucayanum* population from Bimini, the Bahamas, has two breeding periods during the year: in fall and spring, when the water temperature is moderate and the animals tend to spawn the same day, one or two days before the new moon (Holland and Holland 2010).

The length of the life cycle is variable from one species to the other: *B. floridae* can reach the adult stage several months after fertilization (Stokes and Holland 1998), whereas a whole year is needed for *B. belcheri* (Zhang et al. 2007) and more than two years for *B. lanceolatum* (Fuentes et al. 2007; Desdevises et al. 2011).

19.3.2 Animals in the Laboratory

For several years now, some research groups have tried to maintain live amphioxus in their laboratories. Two husbandry systems are mainly used for adults (Carvalho et al. 2017), which both consist of small tanks filled with seawater with or without sediment that are either placed in a water bath to stabilize the temperature or not. In both systems, the water is changed regularly by continuous flow or by big volume changes several times per day, and light is applied in order to get a day/night cycle of 24 hours. Less regular water changes have also been reported for inland laboratories without access to fresh seawater (Theodosiou et al. 2011; Benito-Gutierrez et al. 2013). Adult amphioxus in the field feed by filtering the sea water from which they ingest all the particles less than 100 μm in diameter (Ruppert et al. 2000). Studies of stable isotopes and feces showed that they consume a wide variety of organisms, from bacteria to zooplankton and phytoplankton (Chen et al. 2008; Pan et al. 2015). In the laboratory, a mixture of different algae can be efficiently used to feed adults, although they can survive for months without a food supply (Carvalho et al. 2017). Ripe adults of the four main species used for evo-devo studies—*B. floridae*, *B. belcheri*, *B. japonicum* and *B. lanceolatum*—can be induced to spawn in the laboratory in order to obtain gametes for *in vitro* fertilization (Garcia-Fernàndez et al. 2009). The artificial induction of gamete release was first achieved for *B. floridae* using an electric shock, undertaken at the time of the natural sunset on collected adults kept with a light on (Holland and Holland 1989). However, this method was shown to be efficient only on the days the animals collected would have spawned in the field. For *B. lanceolatum*, heat stimulation by increasing the temperature of the water by 4°C 24 to 36 hours before the desired spawning night can be efficiently used to induce spawning (Fuentes et al. 2007). This technique allows working with embryos at any desired day during the breeding season of this species. The same method has been successfully used in the other *Branchiostoma* species, although with apparently less efficiency. Interestingly, some rearing conditions allow us to

obtain ripe animals all through the year for the Asian species *B. belcheri*, which has never been reliably achieved for any other species (Li et al. 2013; Holland et al. 2015).

Once embryos are obtained by *in vitro* fertilization, they can be cultivated easily in Petri dishes filled with seawater and placed in an incubator to control the temperature. The most delicate step in order to keep amphioxus in the laboratory during their whole life cycle is to raise the larva until they metamorphose to reach the juvenile stage. Larva can be raised in Petri dishes given unicellular algae as food until metamorphosis, but this system is time consuming, as the larva must be manually transferred into clean dishes every day under the binocular (Holland and Yu 2004). Another method, used for *B. belcheri* and *B. japonicum*, is to raise the larva in tanks, with or without sediment. Although by using biggest volume, water changes are less frequently required and easier to manage, the survival rate of larva is very low, at best 3–5% (Zhang et al. 2007). Finally, the only *Asymmetron* species for which laboratory rearing conditions have been reported is the *A. lucayanum* population of Bimini (Holland and Holland 2010; Holland et al. 2015). Adults can be kept in the laboratory in the overall same conditions as the *Branchiostoma* species and *in vitro* fertilization undertaken after spawning. However, the larva die after 10 days of culture with only one open pharyngeal slit, and later stages have yet to be obtained in the laboratory (Holland and Holland 2010; Holland et al. 2015).

19.4 EMBRYOGENESIS

Amphioxus embryogenesis was first described by Kowalevsky (Kovalevskij 1867) for the population of *B. lanceolatum* in the Gulf of Naples. After the zygote cell is formed by external fertilization in the water column, a fertilization envelope detaches from the plasmic membrane and grows, preventing polyspermy and protecting the embryo during its early developmental stages, as observed in other species, such as sea urchins (Holland and Holland 1989). Cephalochordates produce oligolecithal eggs (low amount of yolk evenly distributed in the oocyte) of around 80–100 µm diameter (depending on the species) that undergo a first holoblastic cleavage and produce two blastomeres. Each of these blastomeres is able to develop into a full normal embryo after separation (Tung et al. 1958), although it has been shown
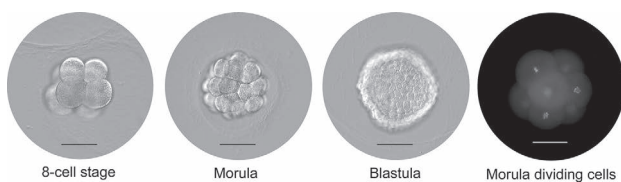
FIGURE 19.2 Cleavage stage. Pictures of *B. lanceolatum* embryos at the eight-cell, morula and blastula stages. During the cleavage period, divisions are synchronous, as shown by the anti-phospho-histone H3 immunostaining of chromosomes in all the cells at the morula stage. Scale bar = 50 µm.
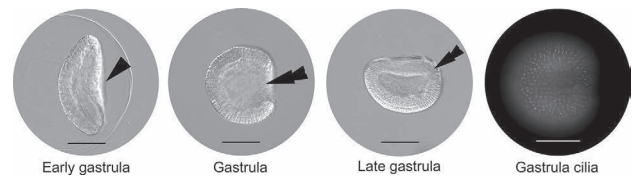

FIGURE 19.3 Gastrulation. Pictures of *B. lanceolatum* embryos during gastrulation. At the beginning of this developmental period, the vegetal plate invaginates (arrowhead) to form the internal layer called the mesendoderm. The opening that is formed is called the blastopore (double arrowheads), which will be completely covered by the epidermis at the end of gastrulation. During gastrulation, cilia grow as shown by anti-acetylated tubulin immunostaining, and the embryo starts to swim. Lateral views with anterior/animal to the left and dorsal to the top. Scale bar = 50 µm.

that at the larva stage, one of the twins develops an abnormal tail (Wu et al. 2011). The second cleavage is perpendicular to the first one, and the third cleavage is unequal, giving rise to the formation of four micromeres at the animal pole and four macromeres at the vegetal pole. After several additional synchronous divisions, the embryo reaches the blastula stage (Figure 19.2).

The blastula corresponds to a single cell layer surrounding a cavity called the blastocoel (Figure 19.2). At this stage, the vegetal region starts flattening and invaginates to form a gastrula with two touching germ layers: the ectoderm (external layer) and the mesendoderm (internal layer) (Figure 19.3). The cavity thus created corresponds to the archenteron, and its opening is called the blastopore. While gastrulation proceeds, cilia grow, and the embryo starts swimming inside the chorion (Figure 19.3).

During gastrulation, contrary to vertebrates, for example, few cells involute, and the two germ layers remain epithelial (Zhang et al. 1997). In the dorsal region, the ectoderm starts to flatten to form the neural plate. The rest of the ectoderm detaches and grows to cover the neural plate and close the blastopore. Before the neural plate is covered, the embryo hatches. Then neurulation proceeds with the neural plate rolling on itself, as observed in vertebrates, to become a hollow neural tube, enlarged in the anterior region, to form the cerebral vesicle. The epidermis that has covered the neural plate fuses in the midline, leaving an opening called the neuropore at the level of the cerebral vesicle (Figure 19.4). At the same time, the dorsal axial region of the mesendoderm starts to form the notochord, whereas in the dorsal paraxial region, pouches pinch off in a segmental manner to form the somites on both sides of the midline (Figure 19.4).

Somites form regularly from the anterior to the posterior region during embryo elongation, first by enterocoely and then by schizocoely from the tailbud. Somites in amphioxus are asymmetric, with the left somites shifted forward by half a somite. At the end of neurulation, the ventral mesendoderm has closed in the dorsal region and forms the future digestive tube. In its anterior region, two diverticula develop (called Hatchek's diverticula) on the right and left sides. The anterior ventral region of the endoderm enlarges to form the future
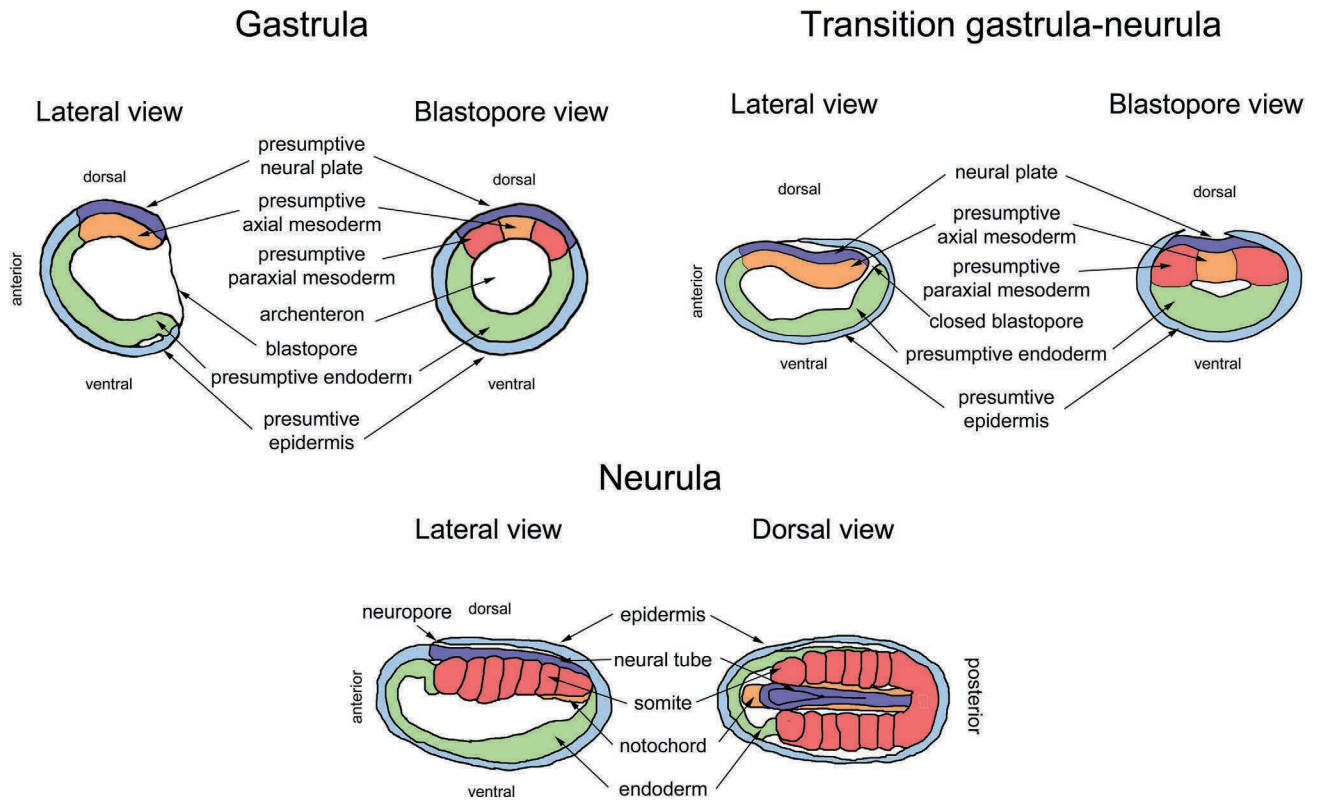
FIGURE 19.4 Diagram of embryos and presumptive fates from gastrula to neurula. Lateral views: dorsal to the top, anterior to the left. Blastopore views: dorsal to the top. Dorsal view: anterior to the left. The ectoderm-derived structures are in blue and light blue, the dorsal mesendoderm-derived structures are in red and orange and the ventral mesendoderm-derived structures are in green.
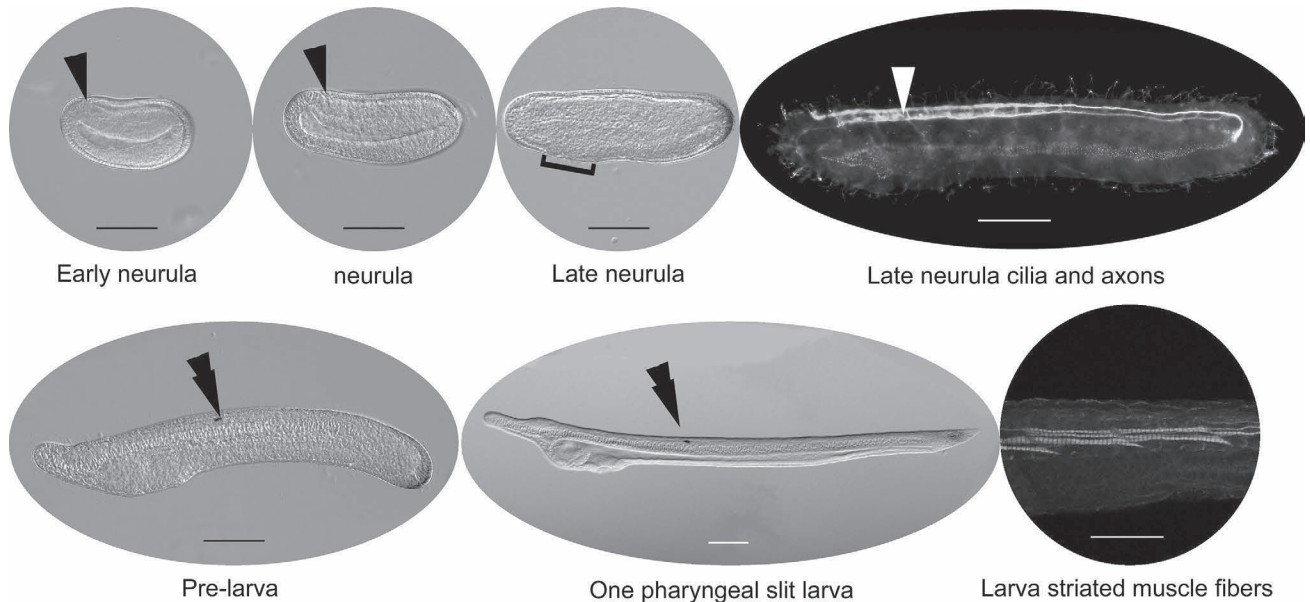
FIGURE 19.5 Neurulation. Pictures of *B. lanceolatum* neurula embryos and larva. At the beginning of the neurulation period, the epidermis has covered the rolling neural plate, leaving an anterior opening at the level of the cerebral vesicle called the neuropore (black arrowhead). In late neurula stage embryos, the pharyngeal region starts to enlarge (bracket) and neurons start to differentiate and grow axons (white arrowhead), as shown by the anti-acetylated tubulin immunostaining. Before the mouth opens, the pigment spot, which is associated with photoreceptor cells, is visible (double arrowhead). In the larva, striated muscle fibers are well developed, as shown by an enlarged picture of a larva after phalloidin-TexasRed labeling, allowing the animal to swim by both muscle contractions and cilia rotation. Lateral views with anterior to the left and dorsal to the top. Scale bar = 50 µm.

pharynx of the larva (Figure 19.5). The first pigment spot, which belongs to a photosensitive organ called the Hesse eyecup, appears. During neurulation, the formed somites elongate in the ventral region. The dorsal part, close to the notochord, forms striated muscle cells, whereas the ventral region participates in the formation of the circulatory system. The ventral region of the first left somite develops into the Hatschek's nephridium, the excretory organ of the larva, whereas the ventral part of the first right somite is considered

a putative hematopoietic region. Finally, the left diverticulum becomes the preoral pit, or Hatschek's pit, and the right diverticulum becomes the rostral coelom, while the endostyle and the club-shaped gland (an organ specific to amphioxus) form from the wall of the pharyngeal endoderm. The mouth opens on the left side and the first pharyngeal slit on the ventral right side of the embryo that becomes a larva (Figure 19.5). At that time, the notochord has grown in the anterior region beyond the cerebral vesicle and segmented striated muscles

(a)

(b)

(c)
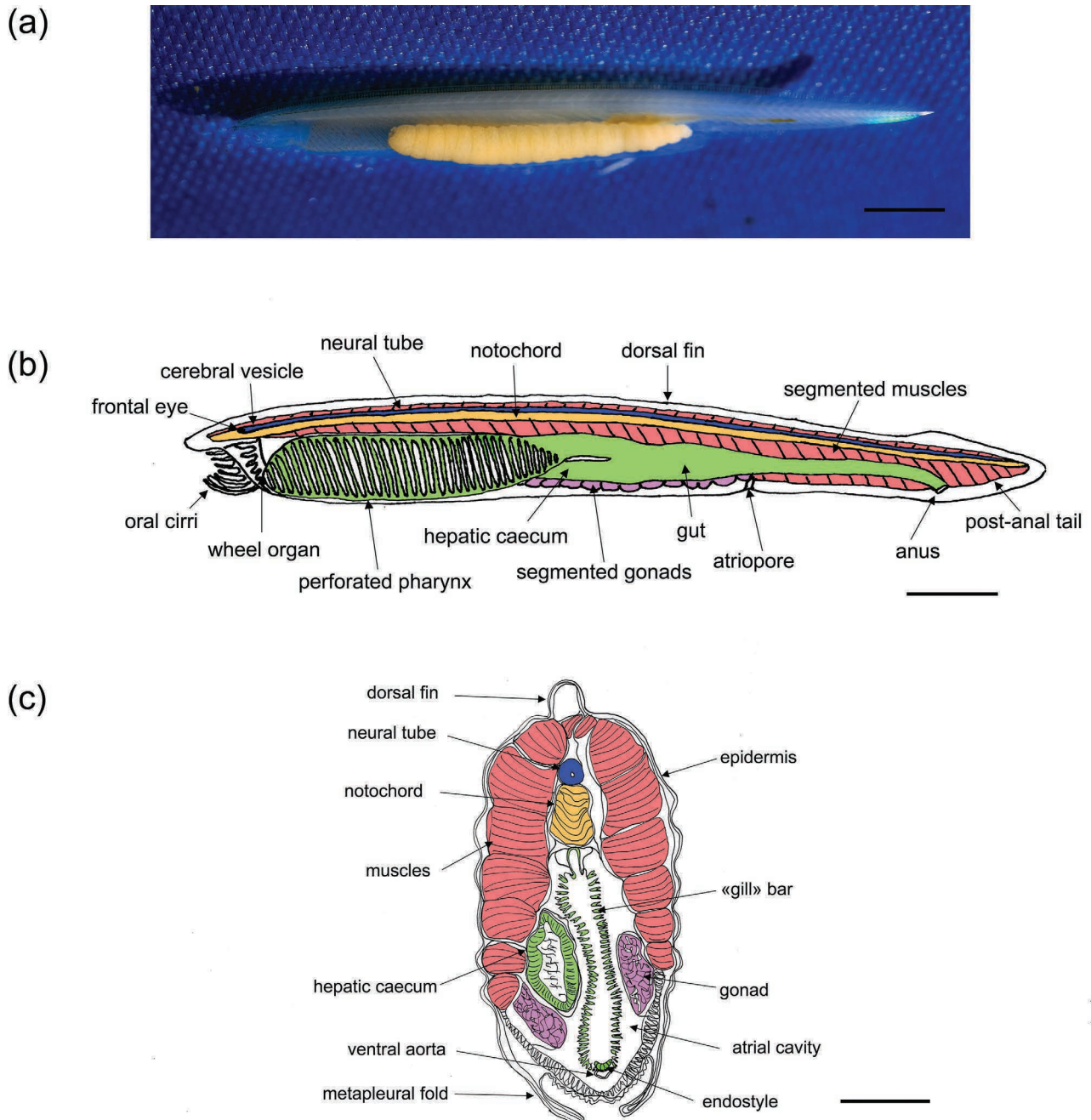
FIGURE 19.6 Morphology of cephalochordates. (a) Picture of an adult amphioxus of the *B. lanceolatum* species with visible gonads. Lateral view, anterior to the left and dorsal to the top, scale bar = 1 cm. (b) Diagram of the morphology of cephalochordates, lateral view with anterior to the left and dorsal to the top, scale bar = 1 cm. (c) Diagram of a cross-section at the level of the pharyngeal region. Dorsal to the top, scale bar = 0.5 cm. ([a] Courtesy of Guido Villani.)

have developed from the dorsal part of the somites, allowing the larva to swim by undulation in the plankton (Figure 19.5). The frontal eye, which is a photosensitive organ proposed to be homologous to the vertebrate retina, forms at the anterior tip of the cerebral vesicle. Finally, the anus opens and the larva starts to feed by filtering the seawater.

After this stage, the larva continues elongating, new somites are still forming in the posterior region and new pharyngeal slits open sequentially posterior to the first one. Once the number of slits has reached a threshold that depends on the species (between 9 and 18) (Holland and Yu 2004; Fuentes et al. 2007; Urata et al. 2007), the larva starts its metamorphosis. This post-embryonic process consists of many morphological modifications. The pharyngeal slits on the right side duplicate and form a second row that migrates toward the left region so that the juvenile possesses a row of slits on both sides of the body. The mouth migrates toward the ventral midline, as well as the endostyle, while the club-shaped gland disappears. Two membranes, called the metapleural folds, grow over the pharynx, cover it and fuse in the ventral midline, forming the atrial cavity that stays open in the posterior region at the level of the atriopore. At the same time, posterior to the pharynx, the hepatic caecum (a digestive gland) starts to bud from the digestive tract. Finally, the cilia of the epidermal cells are lost, and the juvenile migrates to the sediment.

19.5 ANATOMY

The anatomy of amphioxus has been extensively studied since its first scientific description, and a review of all the references can be found in Gans (1996). A diagram of amphioxus anatomy is presented in Figure 19.6. Amphioxus are elongated, almost transparent animals measuring just a few centimeters long at the adult stage. They are characterized by a prototypical chordate body plan and are considered vertebrate-like but simpler (Bertrand and Escriva 2011). As such, they possess a hollow nerve tube in the dorsal region, which forms a cerebral vesicle in the anterior part. Beneath the neural tube is a notochord, which is a rigid rod formed by aligned discoidal cells and which runs more anterior than the cerebral vesicle. This is why they are called cephalochordates ("cephalo" for "head", "chordate" for "notochord", name first proposed by Ernst Haeckel [Nielsen 2012]). The notochord is a shared character among chordates, with tunicates (or urochordates) presenting a notochord in the tail at the embryonic and larval stages at least and vertebrates having an embryonic notochord (except in their most anterior region) that disappears later on during the formation of the vertebral column in almost all species (Stemple 2004; Annona et al. 2015). Ventral to the notochord is the digestive tract: in the anterior region, the mouth is surrounded by oral cirri that form a net able to prevent the entry of big particles into the pharynx. The wheel organ, made of ciliated cells, borders the oral cavity. Posterior to it, the pharynx is windowed thanks to the pharyngeal slits present on both sides of the midline. Posterior to the pharynx are the gut and the

hepatic caecum, the latter of which forms a tongue that is inserted between the pharynx and the wall of the atrium and that opens at the level of the junction between the intestine and the pharyngeal cavity. The ventral wall of the pharynx supports the endostyle, which produces mucus and has been proposed to be homologous to the vertebrate thyroid gland (Ogasawara 2000). Amphioxus swim by undulating their body thanks to the segmented V-shaped muscles that run all along their body on both sides. They also have segmented gonads whose gametes are first released into the atrial cavity and then into the sea water through the atriopore during spawning. The circulatory system consists of several contractile vessels and sinuses, and the vessels are formed by scattered endothelial cells embedded in a basal lamina (Moller and Philpott 1973a, 1973b). The proposed excretory system, although its function still needs to be clarified, corresponds to the Hatchek's nephridium derived from the ventral part of the first left somites and to other nephridia present as a succession of small paired structures associated with the pharyngeal slit clefts (Holland 2017).

19.6 GENOMIC DATA

Genomic and transcriptomic data are powerful resources to pose questions about genomic evolution and genetic control of development. Genomic and transcriptomic data are available for three *Branchiostoma* species (*B. floridae*, *B. belcheri* and *B. lanceolatum)* and transcriptomic data for one *Asymmetron* species *(Asymmetron lucayanum)* (see Table 19.1) (Putnam et al. 2008; Huang et al. 2012; Yue et al. 2014; Marletaz et al. 2018).

19.6.1 BRANCHIOSTOMA FLORIDAE

This was the first genome to be sequenced and assembled in 2008. The project was supported by most of the research groups worldwide working with amphioxus (Holland et al. 2008; Putnam et al. 2008). The *B. floridae* genome was a key contribution to our understanding of chordate evolution and of the origin of vertebrates. It allowed for the reconstruction of the basic gene toolkit involved in development and cell signaling of the last common chordate ancestor. Although it was confirmed that amphioxus mostly contain a single-copy gene for each vertebrate paralogy group and that two rounds of whole-genome duplication predated the vertebrate lineage, it has also been assessed that the amphioxus genome has derived features represented by specific gene family expansion, such as the opsin one (Holland et al. 2008). Moreover, the *B. floridae* genome has allowed a reconstruction of the chromosomal organization of the chordate ancestor. (Access at https://mycocosm.jgi.doe.gov/Brafl1/Brafl1.home.html.)

19.6.2 BRANCHIOSTOMA BELCHERI

The genome of this species was fully sequenced in 2012. The authors developed a novel automated pipeline named HaploMerger to create a better reference haploid assembly

from the original diploid assembly, ensuring better sequence contiguity and continuity (Huang et al. 2012) (Access at http://genome.bucm.edu.cn/lancelet/gbrowser_wel.php.)

19.6.3 *Branchiostoma lanceolatum*

The genome of the Mediterranean amphioxus *B. lanceolatum* was published in 2018. Taking advantage of modern -omics approaches, the efforts of the genome sequencing consortium were focused on the analyses of this species' epigenome. For this purpose, DNA methylation, chromatin accessibility and histone modifications were characterized at the genomic scale. Additionally, transcriptomes across multiple developmental stages and adult tissues were produced. The main conclusion of this study (Acemel et al. 2016; Marletaz et al. 2018) (access at http://amphiencode.github.io), is that the genome of vertebrates has evolved by complexification at different levels, and we will detail this point in Section 19.8.

19.6.4 *Asymmetron lucayanum*

Transcriptomic data from larvae and adults have been generated for *A. lucayanum*, while the whole-genome sequence is not yet available. In their study, by comparing 430 orthologous gene groups among *A. lucayanum*, *B. floridae* and ten vertebrates, Yue and colleagues (2014) showed that cephalochordates are evolving, at the genetic scale, more slowly than any vertebrate, which is consistent with the substantial morphological similarities observed among extant cephalochordates that diverged more than 100 Mya.

19.7 FUNCTIONAL APPROACHES: TOOLS FOR MOLECULAR AND CELLULAR ANALYSES

Classical molecular biology approaches aimed at studying gene and protein localization are feasible in amphioxus, especially in embryos that are completely transparent. In particular, several protocols have been developed for *in situ* hybridization with labeled mRNA probes and for immunostaining approaches using antibodies against endogenous proteins. Moreover, the function of specific signaling pathways has been extensively studied using pharmacological treatments, since amphioxus embryos are particularly suitable for this kind of procedure. Examples on this topic are addressed in Section 19.8.

To understand the function of a given gene, it is necessary to interfere with its correct expression during development. This paradigm is at the base of the functional approaches used in developmental biology research. Classical tools to study gene function are overexpression (by mRNA injection or transient transgenesis), knock-down or knock-out (see Table 19.1). Microinjection is the tool of choice to introduce nucleic acids or proteins into the unfertilized amphioxus egg, rapidly followed by sperm fertilization (Holland and Yu 2004; Liu et al. 2013a; Hirsinger et al. 2015). The redistribution of the injected molecules in daughter cells after mitosis then guarantees gene repression or overexpression during embryonic development. Although there might not seem to be any specific reason for this kind of experiment to be difficult in comparison to similar models as ascidians and sea urchins, the hardness of the chorion and the fragility of the egg make the technique a bottleneck for functional analyses in amphioxus. Overexpression by mRNA injection of certain genes has been successfully achieved in all three main amphioxus species (*B. floridae, B. lanceolatum, B. belcheri*) (Onai et al. 2010; Li et al. 2017; Aldea et al. 2019; Zhang et al. 2019). Gene knock-down has been shown to be effective in *B. floridae* and *B. belcheri* by using gene-specific morpholinos that prevent the translation of mRNAs. Morpholino has been used to study the function of key transcription factors such as *Hox1* and *Pax1/9*, as well as the secreted protein *Dkk3* involved in head specification (Schubert et al. 2005, 2006; Holland and Onai 2011; Onai et al. 2012; Liu et al. 2013b; Liu et al. 2015).

Recently, a genomic mutagenesis approach has been developed in amphioxus by using the transcription activator-like effector nuclease (TALEN)-based technology. This knock-out application to amphioxus boosted the research in

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TABLE 19.1

Availability of tools in different cephalochordate species

	B. floridae	**B. belcheri**	**B. lanceolatum**	**A. lucayanum**
Geographical location	Florida (USA), AO	Asia, PO	Europe, AO + MED	AO + IO + PO
Breeding season	May–September	May–July	May–July	Fall and Spring
Whole life cycle time	3 months	1 year	2 years	N/A
Whole life cycle in the lab	Yes	Yes	N/A	N/A, die at metamorphosis
Whole genome sequence	2008	2012	2018	N/A
Transcriptomes	Embryo larva & adult	Embryo larva & adult	Embryo larva & adult	Larva & adult
Overexpression	mRNA injection	mRNA injection	mRNA injection	N/A
Knock-down/knock-out	Morpholino injection, TALEN	TALEN	N/A	N/A
Transient transgenesis	Yes	Yes	Yes	N/A

AO: Atlantic Ocean, IO: Indian Ocean, PO: Pacific Ocean, MED: Mediterranean Sea

the developmental biology field and filled the gaps with other chordate models (Li et al. 2014). Germ line mutagenesis has been used to study several important developmental genes, such as *Pax1/9*, *Pax3/7*, two ParaHox genes: *Pdx* and *Cdx*, *Hedgehog, Cerberus* and *Nodal* (Li et al. 2014; Wang et al. 2015; Hu et al. 2017; Li et al. 2017; Ren et al. 2020; Zhong et al. 2020; Zhu et al. 2020). Nevertheless, the long life cycle of amphioxus make these and other similar approaches very time consuming; this is the reason the tropical species *B. floridae* is more suitable than temperate species such as *B. lanceolatum*, which takes a few years to reach sexual maturity. It is foreseeable that in the next few years, gene function studies in amphioxus will also take advantage of the genome editing CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/Cas9) technique that represents the next-generation genome editing tool and provides high levels of gene-specific targeting and efficiency.

An efficient transgenic method to study enhancer activity has been recently developed for amphioxus: two transgenic amphioxus lines have been generated using the Tol2 transposon system, based on a hAT family transposon (Shi et al. 2018).

None of these functional approaches have been successfully developed in the *Asymmetron* genus, probably because only a few labs have access to live animals.

19.8 CHALLENGING QUESTIONS

Over the last decades, cephalochordates have become important animal models in the field of evo-devo. The phylogenetic position of amphioxus and its evolutionarily conserved morphology and genome organization make it an extremely useful organism for answering important evolutionary biology questions, in particular with respect to chordate evolutionary history. This section describes some important results obtained using amphioxus as a model as well as key questions for which the full answer is still to be found.

19.8.1 CHORDATE GENOME AND EVOLUTION OF GENOMIC REGULATION

In the 1970s Susumu Ohno, a Japanese-American geneticist, proposed in his book *Evolution by Gene Duplication* that morphological novelties could result from gene duplications and that vertebrate genomes were built by one or probably two whole-genome duplications that took place during the invertebrate chordate to vertebrate transition (Ohno 1970). This hypothesis is named the 2R (for two rounds of duplication) hypothesis. Researchers have long tried to test this assumption using several arguments, such as the number of isozymes, the number of genes or the number of paralogues in vertebrates versus invertebrates. For example, it was shown that amphioxus has a single complete *Hox* gene cluster containing 15 genes, whereas mammals have four incomplete clusters (Amemiya et al. 2008; Putnam et al. 2008). The definitive argument for the 2R hypothesis came with the sequencing of the whole genome of the tunicate *Ciona intestinalis* and was confirmed by the sequencing of amphioxus's genome (Dehal

and Boore 2005; Putnam et al. 2008). Cephalochordates, therefore, have an unduplicated genome compared to vertebrates, and it has been shown that, unlike tunicates, they have retained most of the genes present in the chordate ancestor genome, although some lineage-specific duplications occurred in several gene families (Holland et al. 2008). The cephalochordate genome thus represents the best proxy for the chordate ancestor genome, and analyses of *B. floridae* data allowed the reconstruction of the gene complement of the last common chordate ancestor and the partial reconstruction of its genomic organization (Holland et al. 2008).

Although the evolution of gene content during chordate evolution was probably crucial for their morphological diversification, the contribution of genome architecture and genome regulation is still to be finely studied. In this context, the recent description of the epigenome of the Mediterranean amphioxus, *B. lanceolatum*, already brought new insights. The characterization of the methylome, of chromatin accessibility and of histone modifications at different development stages and in several adult tissues allowed for the discovery of some functional changes that might have given rise to the greater complexity observed in vertebrates (Marletaz et al. 2018). For example, in vertebrates, there has been an increase in regulatory sequences, in particular those that regulate the expression of genes involved in the control of embryonic development. It was also shown that duplicate genes in vertebrates (after the 2R) have evolved mainly by subfunctionalization and specialization and that specialization of gene function was accompanied by an increase in regulatory complexity. Another study, focused on the Hox genomic region, showed that the complex regulation of Hox genes expression in vertebrate is in part due to the acquisition of a new three-dimensional organization of the chromatin around some of the Hox clusters (Acemel et al. 2016). Indeed, the amphioxus Hox gene cluster is contained in a single topologically associated domain (TAD), while in vertebrates, there are two TADs, one on each side of the cluster, and regulatory sequences present in these two TADs are responsible for the regulation of Hox genes expression in the limbs. This study of the *B. lanceolatum* genome also showed that although amphioxus presents a similar pattern of methylation to that of invertebrates (low methylation compared to vertebrates), the expression of some genes is regulated by demethylation in the same way as vertebrates (Marletaz et al. 2018). These recent data pave the way for a better understanding of the genomic regulation principles underlying the morphological and functional innovations of vertebrates. Nevertheless, further effort is necessary to overcome difficulties associated with enhancer element identification and understanding of their functional evolution throughout the last 500 million years.

19.8.2 EVOLUTION OF VERTEBRATE MORPHOLOGICAL TRAITS

Although amphioxus share a typical chordate body plan with vertebrates, they lack key vertebrate characters such as the

head, endoskeleton, migratory neural crest cells, placodes and paired appendages. Therefore, a comparative approach between invertebrate chordates and vertebrates should allow us to discover the main evolutionary innovations that led to the appearance of these complex structures, and amphioxus has been extensively used to answer such questions. In this section, as an example, the contribution of some studies using cephalochordates as a model for our understanding of the evolution of key vertebrate morphological features will be addressed.

19.8.2.1   Cartilage and Bones

One of the most iconic and specific structures of extant vertebrates is their endoskeleton made of cartilage and/or bone that is absent in tunicates and cephalochordates. However, in amphioxus, cartilage-like structures are found at the adult stage in the rods of the cirri that surround the mouth, which consist of cells embedded in a matrix, and in the "gill" bars of the pharynx, which were described as an acellular cartilage (Wright et al. 2001). Although it was proposed that both cartilage-like tissues were non-collagenous (Wright et al. 2001), it has been shown that fibrillar collagen, which is a major component of the cartilage matrix in vertebrates, is present in the pharyngeal "gill" bars (Rychel and Swalla 2007). In search of a conserved gene toolkit for cartilage formation, the expression pattern of amphioxus orthologues of genes controlling cartilage formation in vertebrates has been studied during embryogenesis. No co-expression could be observed, suggesting that cartilage did not appear by co-option of a pre-existing toolkit but probably by the appearance of new gene interactions (Meulemans and Bronner-Fraser 2007). However, these studies were carried out on embryos and not at later stages when the cartilage-like structures form (during metamorphosis). More recent studies using metamorphosing *B. floridae* larvae or regenerating oral cirri in adults have brought new insights on this issue (Kaneto and Wada 2011; Jandzik et al. 2015). It has been shown that during metamorphosis, *ColA*, coding for a collagen in amphioxus, is expressed in the forming oral cirri and in regenerating adult oral cirri as well as transcription factors required for cartilage formation in vertebrates (Kaneto and Wada 2011; Jandzik et al. 2015). The authors also showed that oral cirri formation is dependent upon FGF signaling, a signal which is required in vertebrates for cellular cartilage differentiation, and that adult regenerating cirri rods are expressing genes that are known to be required for osteogenesis in vertebrates (Kaneto and Wada 2011; Jandzik et al. 2015). All together, these data have shown that some elements of the chondrogenic and osteogenic programs of vertebrates were probably already required for the formation of cartilage-like structures in the chordate ancestor. However, more functional data, particularly focusing on amphioxus metamorphosis, are still required to understand the appearance of the vertebrate endoskeleton.

19.8.2.2   Neural Crest Cells

The neural crest cells (NCCs) are a specific transient population of cells specific to vertebrates that are sometimes referred to as the "fourth germ layer" of these animals (Gilbert 2000). They originate from the border of the neural plate at the time at which the neuroectoderm and the future epidermis separate during neurulation (Gilbert 2000). These cells undergo an epithelial-mesenchymal transition, delaminate and migrate all through the body where they differentiate into many different cell types such as melanocytes, adipocytes, neurons, smooth muscles, chondroblasts, odontoblasts and so on (Bronner and Simoes-Costa 2016). NCCs participate in the formation of structures that are vertebrate specific such as bones, cartilage and ganglia of the vertebrate head, and Gans and Northcutt even proposed that the vertebrates' "New Head" (an anterior structure with unsegmented muscles, well-developed brain and sensory organs) appearance was favored by the emergence of NCCs (Gans and Northcutt 1983). In amphioxus, there is no evidence of the existence of such cells, and it is considered that cephalochordates do not have migratory NCCs. However, neurulation occurs in a similar way as observed in vertebrates, and it has been shown that the neural plate border expresses genes that are orthologues of neural plate border specification genes in vertebrates (Yu et al. 2008). On the other hand, among the genes that are known to be required in vertebrates for the specification of NCC or among effector genes (that are downstream of the neural plate border specifying genes in the NCC gene regulatory network), only *Snail* is expressed in the neural plate border of amphioxus (Langeland et al. 1998). Concerning tunicates, the sister group of vertebrates, it has been shown in *Ciona intestinalis* that some cells expressing the NCC specification genes *Id*, *Snail*, *FoxD* and *Ets* differentiate into pigmented cells and that overexpression of *Twist* in these cells induces them to migrate (Abitua et al. 2012), suggesting that NCC would have appeared thanks to the recruitment of a "migratory" program at the neural plate border. However, tunicates have specific developmental modalities among chordates, and cephalochordates seem, at least during early embryogenesis, to develop most of their structures without any step of epithelial-mesenchymal transition, leaving the mystery of NCC emergence still incompletely resolved.

19.8.2.3   Eyes

Among the characters specific to vertebrates, the well-developed pair sensory organs are the most elaborate. The image-forming camera-type eye of vertebrates is a very complex structure composed of different tissues with various embryonic origins. Amphioxus, on the other hand, possess various photoreceptive organs: the lamellar body, Joseph cells, dorsal ocelli and the frontal eye, which is considered homologous to the vertebrate retina (Glardon et al. 1998; Pergner and Kozmik 2017). This very simple organ is formed at the larva stage at the tip of the cerebral vesicle, which is considered homologous to the vertebrate brain. The frontal eye consists of around six photoreceptor cells (Lacalli et al. 1994) of the ciliary type, like the cones and rods of the vertebrate retina, positioned posterior to nine pigment cells (Lacalli et al. 1994). The amphioxus photoreceptors and pigment

cells express genes that are orthologous to genes known to be expressed in the photoreceptor cells and pigmented epithelium of the vertebrate retina, respectively (Vopalensky et al. 2012). Interestingly, other neurons positioned posterior to the row of photoreceptors were proposed to be homologous to the other cell types present in the vertebrate retina: interneurons and/or retinal ganglion cells (Lacalli et al. 1994; Lacalli 1996; Vopalensky et al. 2012). However, data are still missing in order to clearly answer this point. Another important aspect that would support the homology between the amphioxus frontal eye and vertebrate retina is the understanding of the developmental control of frontal eye formation. A recent study showed that, as in vertebrate embryos, inhibiting the Notch signaling pathway during amphioxus embryogenesis increases the number of photoreceptors formed (Pergner et al. 2020), but we are far from a complete understanding of the gene regulatory network underlying the formation of the frontal eye. Another key point that needs to be addressed is how vertebrate paired eyes evolved from a single, midline-positioned ancestral eye.

19.8.3 Evolution of Cell–Cell Signaling Pathways

Harmonious embryonic development relies on the capabilities of cells to communicate in order to construct the correct body plan. For this purpose, they use few signaling pathways, most of them being present in all metazoans (Barolo and Posakony 2002). One important question in the evo-devo field is therefore to understand how the evolution of these pathways (of their actors, roles and interactions) might have participated in the morphological diversification among animals. Amphioxus possess in their genome genes that code for the main actors of all the major signaling pathways, often with one orthologue for several paralogues in vertebrates that resulted from the two whole-genome duplications characterizing vertebrate early evolutionary history (Bertrand et al. 2017). One major issue that needs to be solved is how the multiplication of signaling pathway actors in vertebrates lead to the appearance of their morphological characters. There are still few data concerning this point, but we can cite the case of the retinoic acid receptors (RARs). This transcription factor, which is a nuclear receptor of retinoic acid, is encoded by a unique gene in amphioxus, whereas three paralogues, RARα, β and γ, are found in mammals. By comparing the expression pattern, the function and the binding capacity of vertebrate and amphioxus RARs, it has been proposed that RARβ kept chordate ancestral characteristics, whereas RARα and RARγ acquired new roles (i.e. neofunctionalization) during vertebrate evolution, which might explain the embryonic functions of retinoic acid that are specific to vertebrates (Escriva et al. 2006).

In cephalochordates, the developmental function of many cell–cell communication pathways has been studied mainly thanks to pharmacological treatments capable of inhibiting or activating these signals (for a review, see Bertrand et al. 2017). One of the advantages of using such an approach is the possibility to interfere with signaling pathways at different developmental time windows and therefore to study their implication in diverse developmental processes. Many data obtained in amphioxus have highlighted conservation in the use of different signals for the control of developmental processes with vertebrates, as might be expected given that chordates share a similar body plan. As an example, BMP and Nodal are opposing signals controlling the dorso–ventral patterning of the amphioxus embryo (Onai et al. 2010), the Wnt/β-catenin pathway regulates the formation of the dorsal organizer (Kozmikova and Kozmik 2020) and retinoic acid has been shown to act as a posteriorizing signal and to control the expression of Hox genes (Holland and Holland 1996; Escriva et al. 2002; Schubert et al. 2005), as is the case in vertebrates. However, we can point out some studies that reveal differences between amphioxus and vertebrates that might explain the emergence of some vertebrate novelties. In vertebrates, the somitogenesis process, which consists of the progressive segmentation of the paraxial mesoderm of the trunk during the embryo elongation (Pourquie 2001b), relies on the opposition of two main signals: the retinoic acid differentiating signal in the anterior region that acts in opposition to the fibroblast growth factor (FGF) and Wnt posterior proliferative signals (Pourquie 2001a). In amphioxus, the paraxial mesoderm gets segmented through a similar somitogenesis process, although it is also segmented in the anterior/head region, contrary to what happens in vertebrates. Interestingly, it has been shown in amphioxus that FGF controls only the formation of the anterior somites, that retinoic acid is not involved in this process and that FGF and retinoic acid do not seem to regulate each other during embryogenesis (Bertrand et al. 2011; Bertrand et al. 2015). These results might in part explain how the segmentation of the head mesoderm of vertebrates was lost during evolution and might indicate that the opposition between the FGF and retinoic acid signals, which controls the development of several vertebrate structures, would be a vertebrate novelty.

19.8.4 Evolution of the Immune System

The vertebrate immune system consists of two major components: innate and adaptive immunity. The former is common to all animals, while the latter was believed to be a vertebrate-specific system that relies on lymphocyte cells responsible for the so-called immune long-term memory. Amphioxus genomes possess homologs of most innate immune receptor genes found in vertebrates (Han et al. 2010; Dishaw et al. 2012), and many of these gene families have undergone large lineage-specific expansions, resulting in an extraordinary complexity and diversity of amphioxus innate immune gene complement (Huang et al. 2008). On the other hand, the identification of lymphocyte-like cells in the amphioxus pharynx and the finding of lymphoid proliferation and differentiation genes in cephalochordates indicate the presence of a kind of adaptive immunity system (Huang et al. 2007).

One of the most important events in the acquisition of adaptive immunity in vertebrates was the co-option of the RAG proteins for the antigen receptor gene assembly by V(D)

J recombination. It was long thought that RAG genes evolved from a transposon, and recent data in amphioxus support this hypothesis. Indeed, the amphioxus genome possesses a transposable element called *ProtoRAG* that codes for proteins showing sequence and function similarities with vertebrates RAG1 and RAG2 (Huang et al. 2016). These results highlight how amphioxus immune system studies might bring valuable insights into the evolution of vertebrate immunity.

19.8.5 EVOLUTION OF REGENERATION

Regeneration is a variable feature in chordates, with some species capable of regenerating entire body parts, while others have only reduced abilities to do so. As a result, amphioxus has been shown to be a particularly relevant model organism for our understanding of the evolution and diversity of regeneration mechanisms in chordates. The first observations of this fascinating biological process go back to the beginning of the 20th century, but there has been a revival of interest in this topic in recent years. The latest pivotal studies have highlighted remarkable regenerative features of amphioxus both at the anatomical and molecular levels. In fact, similarities were found between tail regeneration in amphioxus and in vertebrates, although amphioxus can also rebuild the head region, a characteristic that vertebrates have lost (Kaneto and Wada 2011; Somorjai et al. 2012; Somorjai 2017; Liang et al. 2019). Moreover, the regeneration genetic toolkit seems in part to be conserved between amphioxus and vertebrates, as demonstrated by the key role of *Pax*, *Sox* and *Msx* genes (Somorjai et al. 2012; Somorjai 2017) and of the BMP signaling pathway (Liang et al. 2019). Nevertheless, since we are only beginning to dissect the regeneration process in cephalochordates, the potential of amphioxus as a non-vertebrate chordate regeneration model, and to what extent the progress made on understanding the regulation of amphioxus genome may highlight processes that are too complex in vertebrates, remains to be shown.

Importantly, in the last years, evidence of stem cell populations that could contribute to the regenerative process in amphioxus is opening new perspectives. Moreover, recent data suggest the possibility that cephalochordates possess an inherited mechanism for primordial germ cell (PGC) specification rather than an inductive one, as previously thought. PGCs are grouped posteriorly in the endoderm of the neurula tailbud and cluster near the anus at larval stages (Wu et al. 2011; Zhang et al. 2013; Dailey et al. 2016). It is thus very likely that what we will learn from cephalochordate research will complement and help further the study of regeneration and stem cells in vertebrates.

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