

REVIEW

Functional genetic analysis in a jawless vertebrate, the sea lamprey: insights into the developmental evolution of early vertebrates

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ABSTRACT

Lampreys and hagfishes are the only surviving relicts of an ancient but ecologically dominant group of jawless fishes that evolved in the seas of the Cambrian era over half a billion years ago. Because of their phylogenetic position as the sister group to all other vertebrates (jawed vertebrates), comparisons of embryonic development between jawless and jawed vertebrates offers researchers in the field of evolutionary developmental biology the unique opportunity to address fundamental questions related to the nature of our earliest vertebrate ancestors. Here, we describe how genetic analysis of embryogenesis in the sea lamprey (*Petromyzon marinus*) has provided insight into the origin and evolution of developmental-genetic programs in vertebrates. We focus on recent work involving CRISPR/Cas9-mediated genome editing to study gene regulatory mechanisms involved in the development and evolution of neural crest cells and new cell types in the vertebrate nervous system, and transient transgenic assays that have been instrumental in dissecting the evolution of *cis*-regulatory control of gene expression in vertebrates. Finally, we discuss the broad potential for these functional genomic tools to address previously unanswerable questions related to the evolution of genomic regulatory mechanisms as well as issues related to invasive sea lamprey population control.

KEY WORDS: CRISPR/Cas9, Neural crest, Gene regulation, Lamprey, Evo-devo

Introduction: the origin and early evolution of the vertebrates – a developmental perspective

For almost 200 years, the origin and early evolution of the vertebrates has remained a central issue in the fields of comparative biology and natural history (for recent reviews see Gee, 1996, 2018). Throughout much of the 19th and early 20th centuries, the field of comparative embryology strongly influenced studies on vertebrate evolutionary history. Early work by Haeckel, Müller, Kowalevsky, Balfour, Garstang, and others, represented some of the earliest attempts to resolve vertebrate origins within the framework of Darwinian evolutionary theory (Balfour, 1875, 1880; Dohrn, 1875; Garstang, 1894, 1896; Gegenbaur, 1878; Geoffroy Saint-Hilaire, 1830; Haeckel, 1860; His, 1868; Kovalevskij, 1866; Müller, 1869). However, with the rediscovery of Mendelian genetics in the early 20th century, and a shift toward an increasingly quantitative, gene-centric view of biology,

evolutionary embryology soon gave way to population genetics and the ‘modern synthesis’ as the new foundations for evolutionary biology (Bowler, 1989; Dobzhansky, 1937; Fisher, 1930; Huxley, 1943; Needham, 1959). Consequently, the aims of evolutionary embryologists, including the search for and reconstruction of putative vertebrate ancestors, as well as other types of macroevolutionary change, were viewed as anachronistic by a new generation of biologists working under the modern synthesis, compared with the more rigorous statistical approaches offered by evolutionary genetics (Amundson, 2005; Laubichler and Maienschein, 2008; Love and Raff, 2003; Pigiucci and Muller, 2010; Raff, 1996; Wilkins, 2002). Yet, in the past 30 years the fusion of molecular biology, genetics, embryology and modern evolutionary theory – referred to as evolutionary developmental biology, or ‘evo-devo’ – has brought embryology once again to the fore of evolutionary studies (Carroll, 2000; Carroll et al., 2005; Gilbert, 2003a,b, 1996; Hall, 2012; Love and Raff, 2003; Raff, 1996; Wallace, 2002). The field of evo-devo research has offered a wealth of new and exciting findings showing how changes in developmental-genetic programs over time can drive the evolution of morphological, physiological and behavioral adaptations, as well as the origin and evolution of animal body plans, including that of the vertebrates.

The vertebrate body plan can be thought of as a developmental patchwork of phylogenetically integrated parts. Several of these parts can be traced back to the last common chordate or even deuterostome ancestor and have served as a scaffold upon which many new characteristic vertebrate features have evolved. Others appear abruptly in the vertebrate lineage with no obvious forerunners found among invertebrates, whether extinct or extant (Lowe et al., 2015; Satoh, 2016). The list of characters diagnostic of vertebrates is vast (for a recent inventory, see Gee, 2018), but perhaps the most widely recognized of these are: a large tri-partite brain, including the vertebrate-specific telencephalon; a sophisticated peripheral nervous system with paired sensory organs; an inner ear with vestibular apparatus and semicircular canals; a muscular pharynx for pump-based respiration; a head skeleton of cartilage and/or bone; epibranchial, hypobranchial and external eye muscles; a chambered, muscular heart; pharyngeal arteries supported by endothelium; and a segmented renal filtration system, among others (Gee, 1996, 2018; Janvier, 1996b, 2003; Kardong, 2002).

One of the most fascinating features about the vertebrates is that many of their hallmark traits are derived largely from a single embryonic cell population known as the neural crest (for comprehensive reviews, refer to Hall, 2008; Le Douarin and Kalcheim, 1999; Sauka-Spengler and Bronner-Fraser, 2008; Sauka-Spengler et al., 2007; Trainor, 2013). The neural crest is a vertebrate-specific, migratory stem cell population that gives rise to many

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structures that define much of what it means to be a vertebrate, including most of the peripheral sensory nervous system, pigmentation, parts of the heart and teeth, as well as the vertebrate ‘new head’ – the cartilage and bone that form the craniofacial skeleton (Gans and Northcutt, 1983; Green et al., 2015; Northcutt, 2005; Northcutt and Gans, 1983). In gnathostome (jawed) vertebrates, the head skeleton has been substantially modified to give rise to articulated biting jaws that bear rows of sharp teeth (Brazeau and Friedman, 2015; Gans and Northcutt, 1983; Green et al., 2015; Kuratani, 2004; Miyashita, 2016; Northcutt, 2005). All of these features allowed early vertebrate fishes to colonize new ecological niches and acquire novel life history features, such as new and diverse modes of feeding, including active predation in some lineages (Denison, 1961; Gans and Northcutt, 1983; Janvier, 1996b; Mallatt, 1984a,b, 1985; Purnell, 2002). This process led to vertebrates distinguishing themselves morphologically, physiologically and behaviorally from their closest relatives, the invertebrate chordates (Gans and Northcutt, 1983; Northcutt and Gans, 1983). Even now, the neural crest is implicated in the continual morphological evolution among recent vertebrate groups (Fondon and Garner, 2004; Prescott et al., 2015; Sánchez-Villagra et al., 2016; Wilkins et al., 2014). What all of this reveals is that the neural crest not only is responsible for helping to shape much of the vertebrate body plan but also continues to serve as a potent source for the developmental evolution of novel traits.

Jawless vertebrates as models to study vertebrate developmental evolution

The choice of traditional versus non-traditional model systems

How did many of the key features that characterize vertebrates, such as the neural crest, arise? And what are their molecular, cellular and genetic origins in the embryo? To begin to address these types of questions, it is important first to emphasize the significance of the model system that one chooses to work with. Much of our knowledge of vertebrate embryonic development comes from model systems (e.g. mouse, chick, zebrafish, frog; Gilbert, 2006) that enable fine-scale dissection of embryonic development. This is due in large part to the fact that these systems (1) are amenable to the establishment of genetic lines or are at least available throughout most of the year for experiments; (2) have high-throughput biochemical and molecular tools readily available; (3) have high-quality, fully annotated genomes, transcriptomes, etc.; (4) are

capable of consistently yielding embryos for experimental work; and (5) have well-described and vetted protocols for successful and efficient maintenance of adults and embryos.

Answering questions of evolutionary origin, however, often requires a completely different approach and set of criteria (Hall, 2012, 1999; Wallace, 2002). In the past several years the field of vertebrate evo-devo has witnessed an explosion of research into so-called ‘non-model’ systems, including several chondrichthyans (sharks and other cartilaginous fishes), actinopterygians (ray-finned fishes) and agnathan (jawless) fishes – lampreys and hagfishes (Fig. 1) (Adachi et al., 2016; Braasch et al., 2015; Dahn et al., 2007; Gillis and Hall, 2016; Gillis and Tidswell, 2017; Green and Bronner, 2014; McCauley et al., 2015; Modrell et al., 2017a,b; Oisi et al., 2013a,b, 2007; Ota and Kuratani, 2007; Pasquier et al., 2017; Shapiro et al., 2004; Tarazona et al., 2016). Unfortunately, these animals often lack one or more of the features (described above) that make the mainstream developmental models appealing to most embryologists. Why focus on these systems then? Far from being ideal for insights into developmental mechanisms per se, their appeal is instead based largely on phylogenetic position. Each occupies an important node in vertebrate phylogeny and is therefore ideally suited for addressing specific evolutionary questions. For example, sharks are useful for understanding the evolution of dermal skeleton and paired fins; paddlefish and gar (basal actinopterygians) provide insight into the fin-to-limb transition and evolution of special sense organs (Adachi et al., 2016; Dahn et al., 2007; Gillis et al., 2017, 2013, 2012).

The cyclostomes: lampreys and hagfishes

For questions concerning the origin of the vertebrates and vertebrate-specific traits, the ideal models are lampreys and hagfishes (Fig. 1; Shimeld and Donoghue, 2012). Historically, the phylogenetic relationships among hagfishes, lampreys and gnathostomes have been controversial, with competing hypotheses placing lampreys as sister to gnathostomes with hagfishes as an outgroup (agnathan paraphyly), or a grouping of hagfishes and lampreys together as sister to gnathostomes (cyclostome monophyly) (Hardisty, 1982; Heimberg et al., 2010; Janvier, 1996a; Løvtrup, 1977; Miyashita et al., 2019a; Yalden, 1985). Resolution of this issue has important implications for vertebrate evolutionary biologists because each hypothesis has a very different take on the nature of early vertebrates and the assembly and

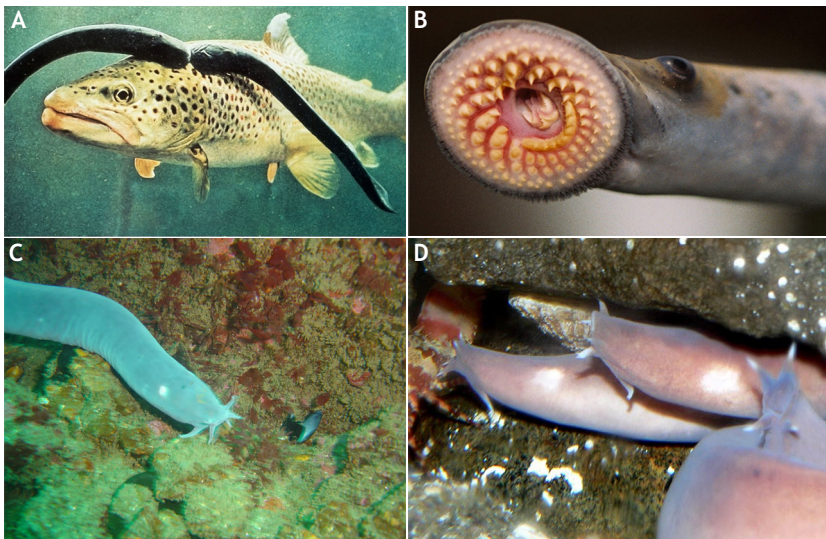


Fig. 1. Jawless vertebrates. Lampreys (A,B; *Petromyzon marinus* pictured) and hagfish (C, *Eptatretus hexatrema*; D, *Eptatretus stoutii*) are the only surviving jawless vertebrates or ‘agnathans’. Images used with permission from Wikipedia commons.

modification of the vertebrate body plan. Current evidence from molecules and morphology now firmly places lampreys and hagfishes together as a monophyletic cyclostome group, originating from an ecologically diverse group of jawless fishes that were some of the first vertebrates to appear on the planet nearly half a billion years ago (Heimberg et al., 2010; Miyashita et al., 2019a,b; Oisi et al., 2013b; Shimeld and Donoghue, 2012; Stock and Whitt, 1992). Unfortunately, lampreys and hagfishes are also the only extant representatives of this group. The rest of the jawless vertebrates and various stem lineages between the cyclostomes and crown group gnathostomes died out over 300 million years ago (Donoghue and Keating, 2014; Donoghue and Purnell, 2005). The importance of the cyclostomes like many other ‘non-model’ systems lies almost entirely in their unique phylogenetic position (Green and Bronner, 2014; Kuratani et al., 2002; McCauley et al., 2015; Medeiros, 2013; Shimeld and Donoghue, 2012). Because they are sister to the jawed vertebrates (gnathostomes), comparison of embryonic development between these two groups allows evolutionary biologists to infer what features were present in the last common vertebrate ancestor (Fig. 2, node ‘a’).

As mentioned above, both lampreys and hagfishes are cyclostomes and form a monophyletic sister group (Fig. 2, node ‘b’) to jawed vertebrates (Fig. 2, node ‘c’). Lampreys and hagfishes are then both, by definition, equally distant from all jawed vertebrates, so in principle each should be equally important for obtaining insight into early vertebrate history. However, this fact ignores important practical concerns that must also be considered. Hagfish embryos have been challenging to obtain because the adults live and spawn in relatively deep sea waters that are difficult to access, their embryos take several months to develop, and they require very precise conditions to live and reproduce in the laboratory (Kuratani and Ota, 2008; Ota et al.,

2007; Shimeld and Donoghue, 2012). To put these difficulties into context, a paper describing hagfish neural crest development (Ota et al., 2007), was the first such description of this animal’s development in over 100 years (Dean, 1899; Kuratani et al., 2016; Kuratani and Ota, 2008; Ota and Kuratani, 2008; Shimeld and Donoghue, 2012).

Lampreys as tractable models for understanding vertebrate developmental evolution

Given the practical bottlenecks that restrict work with hagfishes, much of our knowledge of cyclostome development has instead come from lampreys. Compared with hagfishes, lampreys are much easier to work with. Adult lampreys migrate annually to spawn in shallow streams and rivers in and around North America, South America, Europe, Asia and Australia and are fairly easy to capture (Docker, 2015; McCauley et al., 2015; Potter et al., 2015). Captured sea lamprey can be held in tanks of circulating water maintained at temperatures that have been shown to promote spawning (~18–20°C) (reviewed in Moser et al., 2019). When the animals are mature, gametes can be stripped manually and mixed in small bowls of water, with the resulting embryos being reared successfully at ~18°C (Moser et al., 2019; Piavis, 1961; York et al., 2019).

Nonetheless, there are several features of lamprey biology and life history that have kept them from rising to the status of a traditional model system in developmental biology. One important limitation is that lampreys are seasonal animals and therefore only produce live embryos for experimental biology during a few months in the summer. This obviously limits the scope of investigation. In principle, one can plan to experiment using live animals for the few months they are available annually. In practice, however, variation in sea lamprey egg quality and adult survival often yields only a few

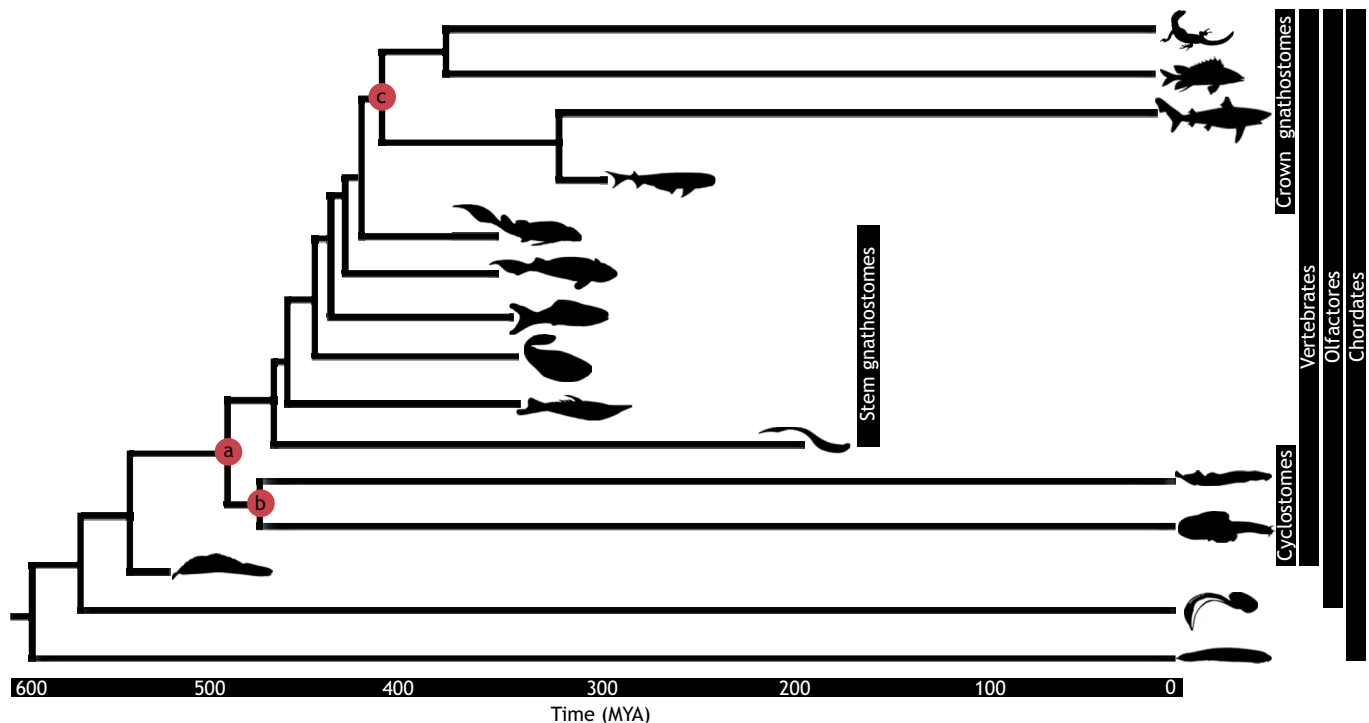


Fig. 2. Lampreys and hagfish occupy a key phylogenetic position for understanding vertebrate evolution. Chordate lineages are diagrammed with approximate divergence times (based on Donoghue and Purnell, 2005). While most early vertebrate lineages diverged from their common ancestor (node ‘a’) around 400–450 million years ago (MYA), only a fraction are extant. In particular, because the stem lineages between cyclostomes (node ‘b’) and crown group gnathostomes (node ‘c’) died out over 300 million years ago, lampreys and hagfishes are the only extant systems we have to study early vertebrate history within a comparative framework. Images are reproduced under creative commons licences from PhyloPic (phylopic.org).

short weeks of access to embryonic stages. We have found that this drawback can be ameliorated in part by keeping immature animals captured in late winter–early spring in chilled holding tanks and then gradually raising the temperature to create a series of maturing animals throughout the summer and early autumn (Moser et al., 2019; York et al., 2019). Other serious issues include the fact that because lampreys are semelparous, the adults cannot be kept for more than a single season for breeding and their larvae take several years to reach maturity (Dawson et al., 2015; Hardisty, 2013; Johnson et al., 2015), and the prolonged post-metamorphic phase of parasitic feeding in sea lamprey in particular exacerbates this problem (Potter et al., 2015). Thus, the unique life history of lampreys in general, and sea lamprey in particular, effectively prohibits the establishment of genetic lines, one of the key advantages of mainstream developmental models.

Because of these difficulties, much of the early developmental work on lampreys was limited in scope. The earliest work on the embryology of lampreys was descriptive in nature, although this soon gave way to experimental techniques such as ablation and transplantation, and, with the advent of evo-devo, techniques such as gene expression analysis via *in situ* hybridization or immunohistochemistry, cell lineage tracing and pharmacological perturbation (Damas, 1943, 1951; Gaskell, 1908; Horigome et al., 1999; Horstadius, 1950; Kuratani et al., 2004; Langille and Hall, 1988; McCauley and Bronner-Fraser, 2003; Murakami et al., 2001; Newth, 1950, 1951, 1956; Nyut, 1955; Tomsa and Langeland, 1999; Ueki et al., 1998). Recently, however, there has been a steady shift toward studying lamprey embryonic development by experimentally determining the function of individual genes or groups of genes (i.e. functional genetic analysis) during lamprey embryogenesis, a goal aided largely by sequencing and annotation of the sea lamprey somatic and germline genomes (Smith et al., 2013, 2018). Although there are many different techniques available for functional genetic analysis, these tools can be grouped broadly into two categories based on how gene function can be manipulated in the embryo: (1) gain-of-function, in which a reagent causes increased or ectopic activity of the targeted gene or gene product (e.g. RNA, protein), and (2) loss-of-function, in which a reagent causes a reduction in, or loss of, activity of the gene or gene product. Below, we describe several different tools, involving both gain- and loss-of-function, that have been used to better understand the developmental genetics of lamprey embryonic development.

Functional genetic analysis of lamprey development

Early approaches: antisense genetic tools

The development of genetic tools such as antisense morpholino oligonucleotides (morpholinos) in the early 2000s was an important step forward in unraveling the genetic control of embryonic development (Blum et al., 2015; Nasevicius and Ekker, 2000; Summerton and Weller, 1997). By binding to the start codon or splice site junction of a nascent messenger RNA (mRNA), morpholinos inhibit the translation or processing of the target transcript, effectively ‘knocking down’ gene function in the embryo (Blum et al., 2015; Nasevicius and Ekker, 2000; Summerton and Weller, 1997). McCauley and Bronner-Fraser (2006) were the first to use morpholinos in a lamprey species (*Petromyzon marinus*) by targeting the transcription factor *SoxE1* to understand its functional role in neural crest and pharyngeal arch development. This was followed up by work in the Bronner lab by Sauka-Spengler et al. (2007) and Nikitina et al. (2008, 2011), who used morpholinos to demonstrate that much of the neural crest gene regulatory network

(GRN) is conserved between lampreys and gnathostomes. Finally, Lakiza et al. (2011) used morpholinos to tease apart the specific roles of *SoxE1*, *SoxE2* and *SoxE3* genes during development of the embryonic and larval head skeleton.

While microinjection of morpholinos has been useful for studying lamprey embryonic development, this technique also suffers from substantial drawbacks. Morpholinos have relatively high sequence specificity, but they are also expensive, making multi-gene interrogation cost-prohibitive for many laboratories. This can be problematic when moving from single-gene analysis to testing the roles of multiple genes and their interactions within developmental GRNs. Another concern is that morpholinos can only cause transient loss of gene expression (knockdown), rather than permanent genomic knockout (Bedell et al., 2011; Eisen and Smith, 2008). Because lamprey embryos develop much more slowly than other vertebrates (Nikitina et al., 2008; Square et al., 2015), this can present problems when studying the effects of gene knockdown on embryonic development at later stages (e.g. ammocoete larvae) because of possible effects of reagent dilution over time. In sum, although morpholinos initially proved useful, the rather limited scope of their application, as well as several practical hurdles, led to an eventual impasse in the ability of researchers to move beyond simple knockdown experiments involving one or a few genes to a more integrated and mechanistic understanding of genomic regulation during lamprey development.

CRISPR/Cas9 – a powerful new tool for genetically dissecting lamprey embryonic development

Many of the problems presented by morpholinos have been overcome through the use of the Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) system. CRISPR/Cas9 is a revolutionary genome editing technology that has paved the way for significant advances in functional genomics in almost any developmental model, including non-traditional models such as lampreys. The CRISPR/Cas9 system evolved as an adaptive immune defense in bacteria and archaea against viral DNA insertion into the genome, and has been adapted for laboratory use across a wide range of eukaryotes (Doudna and Charpentier, 2014; Garneau et al., 2010; Horvath and Barrangou, 2010; Kunin et al., 2007; Qi et al., 2013; Sander and Joung, 2014; Wang et al., 2016). Given the remarkable capacity for CRISPR to edit with high precision almost any region of an organism’s genome, it is perhaps equally remarkable that the system itself is relatively simple, in terms of both the number of components required and the mechanism of action. The ‘standard’ system used routinely for genomic DNA modification involves the Cas9 nuclease derived from *Streptococcus pyogenes* as well as a short guide RNA (sgRNA) (Doudna and Charpentier, 2014; Garneau et al., 2010; Horvath and Barrangou, 2010; Kunin et al., 2007; Qi et al., 2013; Sander and Joung, 2014; Wang et al., 2016). When delivered into zygotes or individual cells, the Cas9 protein complexes with the sgRNA and, when bound to the targeted sequence, induces a double-stranded DNA break that may be repaired by non-homologous end joining (NHEJ) repair machinery. Because NHEJ repair is error prone, this can result in short insertion/deletions (indels) that generate embryonic mutant genotypes that can be screened for phenotypic effects (Guo et al., 2014; Hwang et al., 2013; Irion et al., 2014; Jao et al., 2013; Qi et al., 2013; Square et al., 2015; Wang et al., 2016; Zu et al., 2016).

CRISPR/Cas9 genome editing in sea lamprey – proof of principle

The first application of the CRISPR/Cas9 system for gene editing in sea lamprey was a proof-of-principle study led by the Medeiros

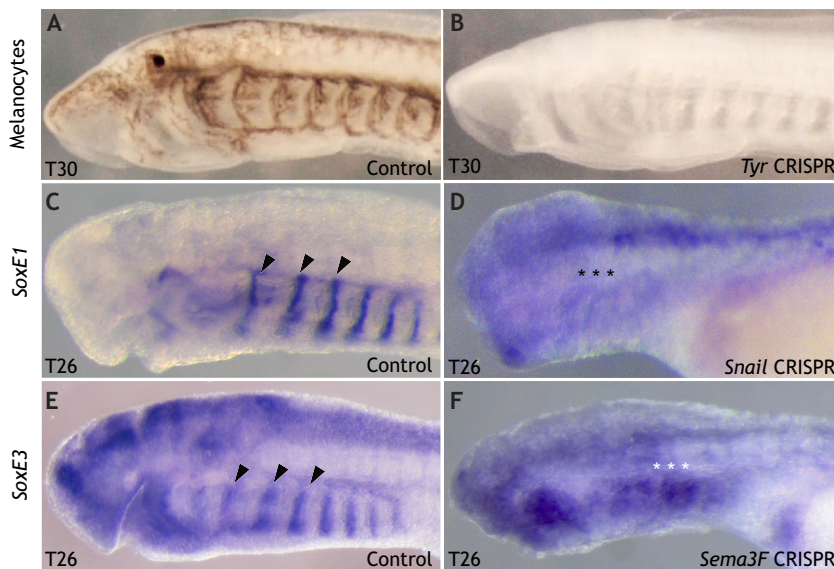


Fig. 3. Using CRISPR/Cas9 to study embryonic development and evolution in sea lamprey.

(A,B) CRISPR-mediated loss of *Tyrosinase* (*Tyr*) activity resulting in loss of melanocytes (brown pigment spots) yields an albino phenotype. (C,D) Mutations induced at the *Snail* locus result in failure of *SoxE1*-expressing pharyngeal cartilage to form (compare arrowheads in C with asterisks in D). (E,F) Mutagenesis of *Sema3F/Nrp* signaling prevents proper patterning of *SoxE3*-expressing pharyngeal cartilage bars into a segmental morphology (compare arrowheads in E with asterisks in F). See text for details and associated references. T26, Tahara stage 26 embryo; T30, Tahara stage 30 ammocoete larva (Tahara, 1988). Original images in A and B were kindly provided by D. Medeiros (Department of Ecology and Evolutionary Biology, The University of Colorado at Boulder). Original images in C–F are from experiments performed by J.R.Y.

laboratory. Square et al. (2015) began by targeting *Tyrosinase* (*Tyr*), a gene in the melanin synthesis pathway in melanocytes. Knockout of *Tyr* is useful for demonstrating proof of principle because pigment presence/absence is an easy phenotype to score. Square et al. (2015) found that two independent sgRNAs consistently caused reduction or loss of pigment in larvae (Fig. 3A,B). After demonstrating that CRISPR could be used to efficiently edit the genomes of sea lamprey embryos, they next sought to knock out genes known to regulate key aspects of vertebrate embryogenesis. To this end, they designed sgRNAs targeting FGF8/17/18, a ligand component of canonical fibroblast growth factor (FGF) signaling. These knockouts revealed key regulatory functions for FGF signaling in sea lamprey that parallel those described in jawed vertebrates, including patterning of somites, pharyngeal arch outpocketing and head skeleton development (Abu-Issa et al., 2002; Crump et al., 2004; Jandzik et al., 2014; Wilson and Tucker, 2004). The Medeiros group also found that perturbation of FGF signaling caused loss of *HhA* expression in the zona limitans intrathalamica and *Engrailed* expression at the midbrain–hindbrain boundary, both of which are key organizing centers in the embryonic brain (Sugahara et al., 2011). A second demonstration of CRISPR/Cas9 in sea lamprey was by the Li laboratory, which focused on evaluating the efficacy of CRISPR knockouts on several genomic loci, including *Golden* (*Gol*), *Kctd10*, *Wee1*, *SoxE2* and *Wnt7b* (Zu et al., 2016). Overall, and similar to Square et al. (2015), Zu et al. (2016) found high rates of biallelic mutagenesis, albeit with modest variation in efficacy across genomic targets and with different sgRNAs.

CRISPR/Cas9 for studies of lamprey development and vertebrate evolution

After establishing that the CRISPR system could in fact be used to study sea lamprey development, a string of papers has been published recently from the McCauley laboratory showing the utility of CRISPR as an important functional genomic tool for testing important hypotheses relating to the evolution of the vertebrate neural crest and the evolutionary origin of new cell types in the vertebrate nervous system.

The first of these came with a study by York et al. (2017) examining the ancestral mechanisms regulating neural crest specification and migration. In jawed vertebrates, neural crest cells

originate in the dorsal neural tube, but must eventually segregate from the rest of the cells in the neural tube that will form the central nervous system proper (Fig. 4; Betancur et al., 2010; Kerosuo and Bronner-Fraser, 2012; Sauka-Spengler and Bronner-Fraser, 2008; Savagner, 2010). This process is mediated in part by differential expression of cadherin cell surface adhesion proteins: neural crest cells upregulate type II cadherins and downregulate type I cadherins; the rest of the neural tube suppresses type II cadherins while strongly expressing type I cadherins (Gheldof and Berx, 2013; Park and Gumbiner, 2012; Rogers et al., 2013; Savagner, 2010; Taneyhill and Schiffmacher, 2013). This so-called ‘cadherin switch’ is mediated by the transcription factor *Snail*, which directly represses type I cadherin loci and stimulates expression of type II cadherins (Bolos et al., 2016; Molina-Ortiz et al., 2012; Schiffmacher et al., 2014; York et al., 2017). The result is the breaking of physical ties between the neural crest and neural tube, setting into motion gene regulatory activity promoting migration. Although cadherin switching is evolutionarily conserved among jawed vertebrates, it was unknown whether this phenomenon also occurred in jawless vertebrates.

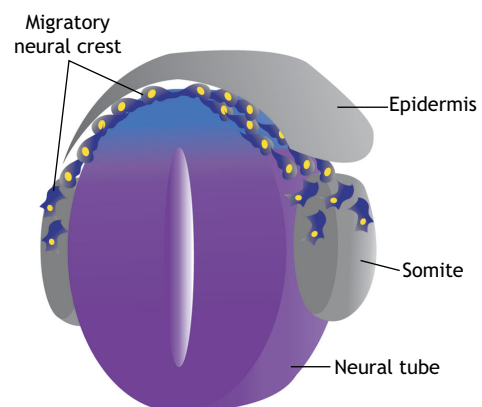


Fig. 4. Schematic cross-section showing migration of neural crest cells from the dorsal neural tube of a generalized vertebrate embryo.

Premigratory neural crest cells (blue shading) are specified in the dorsal-most aspect of the neural tube. Soon after specification, these cells delaminate from the underlying neural epithelium and then begin to migrate laterally and ventrally, and in doing so invade surrounding tissues.

York et al. (2017) addressed this by using CRISPR to examine the functional role of *Snail* in the context of neural crest migration and cadherin switching. Surprisingly, they found that sea lamprey embryos do not use cadherin switching to initiate neural crest migration. Instead, lamprey embryos simply upregulate expression of a pro-migration type II cadherin without changing expression of a pro-epithelial type I cadherin. By contrast, CRISPR knockouts revealed that lamprey *Snail*, as in other vertebrates, was indeed necessary for initiating neural crest migration. This occurs in lamprey by *Snail*-mediated activation of type II cadherin expression, as well as *SoxE* genes in the premigratory and migratory neural crest. CRISPR-mediated loss of *Snail* activity therefore inhibits migratory neural crest cells from colonizing the pharyngeal arches, resulting in a failure of pharyngeal cartilage to form (Fig. 3C,D). These results point to an important functional role for *Snail* in the ancestral vertebrate neural crest GRN, but also highlight important differences in the use of cadherins to control neural crest migration.

York et al. (2018) subsequently used CRISPR/Cas9 to explore the ancestral basis for neural crest patterning mechanisms. This study focused specifically on *Semaphorin3F/Neuropilin* (*Sema3F/Nrp*) signaling, because although it patterns the neural crest in jawed vertebrates, it was unknown whether a comparable signaling system operated in jawless vertebrates (Berndt and Halloran, 2006; Gammill et al., 2007; Osborne et al., 2005; Yazdani and Terman, 2006; York et al., 2018; Yu and Moens, 2005). York et al. (2018) showed that lamprey embryos deploy *Sema3F/Nrp* signaling during all phases of neural crest development, and that this pathway is essential for the organization of migratory and post-migratory neural crest cells into key vertebrate features, including pigmentation patterns, the peripheral sensory nervous system and, in particular, the head skeleton (Fig. 3E,F). This result suggests that the evolution of *Sema3F/Nrp* signaling was pivotal because it allowed early vertebrates to pattern groups of neural crest cells into specific morphological structures that are functionally important for vertebrate biology. However, York et al. (2018) also found that *Sema3F/Nrp* activity was not necessary for the segregation of migratory neural crest streams or patterning of trunk neural crest derivatives, such as dorsal root ganglia or gut neurons. This raised the possibility that *Sema3F/Nrp* signaling in trunk neural crest may have evolved after the split of the cyclostome and jawed vertebrates.

Finally, Yuan et al. (2018) took advantage of CRISPR to address the origin of the genetic mechanisms required for production of vertebrate glial cells, the cells that surround neurons to provide structural support and insulation (Yuan et al., 2018). One important glial subtype includes oligodendrocyte precursor cells (OPCs) which, among other things, give rise to myelinating glial cells within the vertebrate nervous system. OPC gliogenesis is tightly regulated in the ventral neural tube by an evolutionarily conserved suite of transcription factors, which include *SoxE*, *PDGF-R* and *Nkx2.2* (Baroti et al., 2016; Fu et al., 2002; Stolt et al., 2006; Yuan et al., 2018). Through a combination of cross- and auto-regulatory activity, these genes specify OPC identity, with downstream genes such as myelin basic protein (MBP) and proteolipid protein (PLP) being activated to drive OPC differentiation (Qi et al., 2001; Zhu et al., 2014).

The evolution of OPCs is significant because extant cyclostomes lack myelin, but it was unknown whether these animals possessed a non-myelinating OPC precursor cell type. Assuming that the cyclostome condition reflects that of ancestral vertebrates (i.e. no secondary loss of myelinating OPCs), there are at least three different evolutionary scenarios for OPC and myelin evolution:

(1) non-myelinating OPCs may have evolved in ancestral vertebrates, with a myelinating function appearing later in jawed vertebrates; (2) OPCs and myelin evolved contemporaneously, but only in jawed vertebrates; and (3) OPCs and myelin appeared along stem lineages between cyclostomes and crown group gnathostomes. To test these possibilities, Yuan et al. (2018) identified lamprey homologs of each of three key transcription factor families required for OPC development (*SoxE1* and *SoxE3*, *PDGFRab* and *Nkx2.2*), and found that all were expressed in overlapping patterns in the ventral neural tube during gliogenesis – similar to that of OPC development in jawed vertebrates. Using CRISPR knockouts, they further demonstrated that these transcription factors display regulatory interactions reminiscent of those in jawed vertebrate OPCs and that their activity in the ventral neural tube is functionally required for glial differentiation. These results raise the possibility that OPCs may be present in lamprey and thus could have evolved at the base of vertebrates without performing a myelinating function. The implication of this is that myelination may have evolved after the appearance of OPCs along stem lineages leading to jawed vertebrates by the insertion of differentiation genes such as MBP and PLP downstream of an ancestral *SoxE–Nkx–PDGFR* regulatory axis.

In summary, these studies show that CRISPR is a relatively easy system to implement in sea lamprey, and presumably other lamprey species, and has already started to provide important insights into the nature of early vertebrate biology. In the next section, we describe different ways in which CRISPR can be applied to study numerous other aspects of lamprey embryonic development, including *cis*-regulation of gene expression, gain-of-function/overexpression, and genomic knock-in and gene replacement experiments.

Potential applications of CRISPR in lampreys

Genetic dissection of *cis*-regulatory elements

Currently, the use of CRISPR-based functional genomic tools to study lamprey development (see above) has consisted of knockouts of genomic protein coding sequences. This approach has been useful for studying gene function during embryogenesis and identifies epistatic (genetically ‘upstream’) or hypostatic (genetically ‘downstream’) gene relationships within developmental GRNs. Unfortunately, these data tell us relatively little regarding exactly how, when and where particular genes become activated (or repressed) during embryonic development. The spatial and temporal expression of any gene in an organism’s genome is ultimately dependent upon gene-specific control regions in genomic DNA, referred to as non-coding *cis*-regulatory DNA elements (CREs; for comprehensive reviews, see Britten and Davidson, 1969; Davidson, 2001; Davidson, 2010b). Because gene expression can be reduced directly to sequence-dependent control by CREs, and evolutionary change is governed largely by the control of gene expression, one of the goals of modern evo-devo research is to understand how CREs control gene expression and how changes in their ‘hardwiring’ within developmental GRNs lead to evolutionary transitions (Britten and Davidson, 1969; Davidson, 2001a,b; Davidson and Erwin, 2010; Davidson et al., 2002; Hinman and Davidson, 2007).

The lack of reliable technologies to add, remove or otherwise modify genomic CREs has hindered the linkage of CRE regulatory activity to phenotype, especially in non-model organisms. In sea lamprey, a couple of different methods have been tested to begin to study CRE function *in vivo*. These have included transient transgenesis of circular or linearized reporter DNA, Tol2-mediated recombination, and integration of putative CREs and reporters into the genome via the homing endonuclease

meganuclease I-SceI (Kusakabe et al., 2003; Parker et al., 2014b). The meganuclease method has by far been the most successful. This technique yields the greatest survivorship with a high degree of consistent and uniform expression (i.e. low mosaicism) and has been used with success to study the *cis*-regulatory control of *Hox* gene expression during patterning of the embryonic hindbrain and neural crest (Parker et al., 2014a,b, 2019). Parker et al. (2014a, 2016) found that, even though lampreys lack obvious morphological segmentation in the form of hindbrain rhombomeres characteristic of jawed vertebrates, the underlying ‘molecular anatomy’ for rhombomeric segmentation via *Hox*, *Kreisler* and *Krox20* is nonetheless present in lamprey embryos. Most recently, they used meganuclease-mediated transgenesis to demonstrate that neural crest regulatory elements in gnathostome embryos (e.g. *Crestin* in zebrafish) can mediate reporter expression in lamprey embryos (Fig. 5). Parker et al. (2019) also used these assays to show that ancestral vertebrates likely had single *Hox* enhancers for hindbrain and neural crest patterning that have subfunctionalized independently in lampreys and jawed vertebrates.

The CRISPR/Cas9 system has been adapted to allow both CRE genomic integration and CRE loss-of-function, and has the potential to provide important insight into the functional genomics of CREs (Fulco et al., 2016; Guo et al., 2015; Korkmaz et al., 2016; Li et al., 2014; Wright and Sanjana, 2016). Going forward, CRISPR/Cas9 could be combined with a variety of genomic tools to test the function of isolated CREs during lamprey development. For example, multiple sequence alignment or genome analysis software can be used to identify putative regulatory sequences based on sequence conservation across taxa (phylogenetic footprinting) (Sandelin et al., 2004; Visel et al., 2007, 2006; Wasserman and Sandelin, 2004). Alternatively, molecular assays such as ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing), can identify areas of open chromatin (a proxy for

putative CREs) in specific tissues and during specific phases of development (Adachi et al., 2016; Buenrostro et al., 2015; Gehrke et al., 2015; Parker et al., 2019). With information about a putative CRE in hand, flanking sgRNAs can excise (or replace, i.e. ‘knock-in’) the entire genomic sequence. Injected embryos can then be analyzed for changes in morphology, as well as gene expression at the locus associated with the CRE qualitatively by *in situ* hybridization and immunohistochemistry (Kvon et al., 2016; Osterwalder et al., 2018). Alternatively, approaches such as quantitative polymerase chain reaction (qPCR) and total RNA-sequencing (RNA-seq) can be used to more precisely track changes in gene expression in response to mutagenesis at the target locus (Dickel et al., 2018; Guo et al., 2015; Korkmaz et al., 2016; Kvon et al., 2016; Li et al., 2014; Osterwalder et al., 2018). Regardless of the exact method used, it is now possible to show how CRE sequence structure directly influences gene expression output during embryonic development in lamprey embryos.

Tissue-specific gene activation

The CRISPR/Cas9 system has also been modified to allow for gain-of-function genetic manipulations. Gain-of-function experiments have usually involved microinjection of a vector containing a constitutively active promoter driving production of complementary DNA (cDNA, i.e. the coding sequence of DNA) of the gene of interest, or *in vitro* transcribed mRNA (Lloyd, 2003; Prelich, 2012). The ultimate goal in either case is the ectopic or excess production of gene product that may cause a change in phenotype (morphology, cell fate changes, alterations of gene regulatory networks, etc.). In contrast, the CRISPR system for gain-of-function relies on a catalytically inactive form of Cas9 (dCas9) fused to a transcriptional activator domain (Gilbert et al., 2014; Maeder et al., 2013; Perez-Pinera et al., 2013). The dCas9-activator is targeted to the promoter of the target gene by the sgRNA. Rather than cleavage, however, binding of dCas9 results in ectopic transcription of the gene in a tissue-specific context. With these tools in hand, it should be possible for lamprey embryologists to precisely overexpress targeted genes for studies of gene regulation.

Genomic knock-in and gene replacement

A powerful approach to studying the evolution of protein function is to overexpress or replace orthologous gene segments or even entire genes between groups of organisms and then evaluate the ability of these constructs to perform the task of the native gene. Lee et al. (2016) took a similar approach by overexpressing sea lamprey *SoxE* genes (*SoxE1*, *SoxE2*, *SoxE3*) in zebrafish *Sox10* mutants (*colourless*) to test the ability of each lamprey paralog to rescue defects in the production of neural crest-derived neurons and melanocytes. They found not only that lamprey *SoxE2*, the paralog of *Sox10*, could rescue mutant phenotypes but also that overexpression of *SoxE2* in wild-type lines resulted in an excess of melanocytes. *SoxE1* and *SoxE3*, by contrast, were not as effective for rescue of melanocyte defects. This suggests that *SoxE2/Sox10* protein function has been maintained by selection despite over 450 million years of independent evolution, but that there has been significant divergence in the function of other *SoxE* genes between cyclostomes and jawed vertebrates.

With CRISPR, it is now possible to perform these and similar experiments more rigorously by using sgRNAs to precisely and permanently replace entire genes or gene fragments (e.g. protein dimerization or DNA binding domains) in jawed vertebrate genomes with their lamprey orthologs and vice versa by genomic knock-in (Auer et al., 2014; Platt et al., 2014). Similar experiments involving

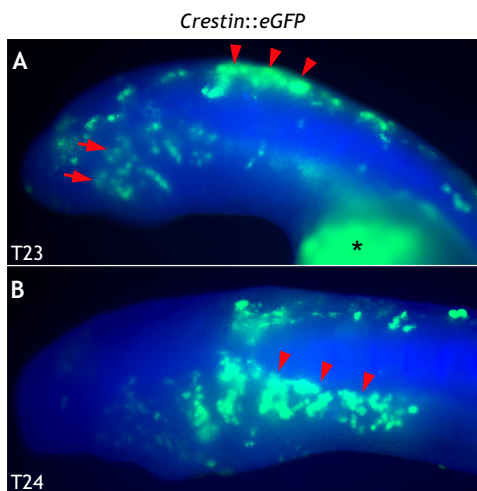


Fig. 5. Meganuclease-mediated transgenesis in lamprey allows dissection of vertebrate *cis*-regulatory DNA elements (CREs). I-SceI meganuclease-mediated transgenesis reveals that lamprey embryos can decode zebrafish neural crest regulatory elements for *Crestin* as indicated by *eGFP* expression (green) in premigratory (arrowheads, A) and migratory neural crest cells (arrows, A) at T23, and in neural crest cells at T24 colonizing the pharyngeal arches (arrowheads, B). The blue color of each embryo is autofluorescence of lamprey tissue visualized using the DAPI channel. Asterisk in A indicates non-specific reporter expression in the yolk. The *Crestin::eGFP* construct was a kind gift from Hugo Parker (Stowers Institute, Kansas City, MO, USA). T23, Tahara stage 23 embryo; T24, Tahara stage 24 embryo (Tahara, 1988). Microinjection and imaging of the construct in lamprey embryos were performed by J.R.Y.

replacement of orthologous enhancer sequences between jawed and jawless vertebrates (e.g. *Hox* genes) could be used to test whether homologous *cis*-regulatory elements can mediate endogenous gene expression across vertebrates.

Gene editing for management of invasive sea lamprey

In addition to the insights they provide for understanding vertebrate evolution, knowledge of the biology of lampreys also has important consequences for management practices. Sea lamprey are an invasive pest species in the Laurentian Great Lakes that border the USA and Canada (Applegate, 1950; Eshenroder, 2014). With completion of the Welland Canal in the 19th century, land-locked sea lamprey in Lake Ontario were able to bypass Niagara Falls, a natural barrier to their upstream migration, and are now present throughout the Great Lakes, where no natural predators exist (Applegate and Moffett, 1955). Parasitic sea lamprey, which feed on blood and fluids from their host fish, devastated the Great Lakes fishery industry in the early 20th century (Marsden and Siefkes, 2019; Siefkes, 2017). Economic losses resulting from sea lamprey parasitism soon led to efforts to control their numbers (Applegate, 1950). Biocontrol efforts have been led principally by the Great Lakes Fishery Commission, established in 1955 by the Convention on Great Lakes Fisheries between the USA and Canada. Currently, sea lamprey in the Great Lakes are managed primarily by using a combination of barriers to spawning migration and lampricides that kill larval lamprey (Siefkes, 2017). The long-term viability of both options is uncertain, however, which has prompted the Great Lakes Fishery Commission to explore other possibilities, including genetic biocontrol. In support of the latter, recent developments in gene editing techniques as described above demonstrate that lamprey gene sequences can be edited efficiently, and other genetic techniques such as RNA interference (RNAi) have been used to demonstrate efficient disruption of gene activity in adults (Heath et al., 2014). Transient expression of tissue-specific reporter genes has also been demonstrated in lamprey, allowing for direct tests of the role of different genes in sea lamprey development. In their review of genetic biocontrol options potentially applicable to sea lamprey, Thresher et al. (2019) listed as a top priority research and development to determine whether it is feasible to use genetic manipulation to heritably bias sea lamprey sex ratios toward the male phenotype, or to decrease the viability of their offspring by interfering with gamete maturation. Although cues to sex determination in lampreys at this stage remain unclear (i.e. environmental versus genetic sex determination) (Docker and Beamish, 1994; Docker et al., 2019; Johnson et al., 2017), those leading to sex differentiation likely depend on gene functions that regulate reproductive physiology. The development and testing of gene editing tools toward perturbing sea lamprey reproductive physiology, for example, by biasing sex ratios and/or reducing fecundity, may offer solutions to this ongoing problem.

Conclusions

Although still in its infancy, functional genomic analysis of embryonic development in jawless vertebrates such as lampreys, through the use of CRISPR/Cas9 genome editing and transient transgenic assays, promises to offer unparalleled insight into the operation of genomic regulatory systems in this phylogenetically important group. Going forward, the application and modification of these tools will prove critical for vertebrate evo-devo biologists to tackle fundamental questions relating to the nature of the earliest vertebrates and how changes in developmental-genetic programs over time have led to their diversification and success.

Acknowledgements

We thank D. Medeiros for providing the image of *Tyr* loss of function in lamprey. We also thank two reviewers for insightful comments to improve the manuscript. This paper emerged from the JEB Symposium 2019: Genome editing for comparative physiology.

Competing interests

The authors declare no competing or financial interests.

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