

Building a Species: Evolutionary Developmental Biology as the Basis for Novel Morphology

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Evolutionary developmental biology introduces a new model of evolution that integrates developmental and population genetics to explain the origin of morphological diversity. The idea that heritable changes in the development of organisms drive evolution is integral to this perspective. Just as evolution creates novel structures from existing parts, evolutionary developmental biology demonstrates that evolution affects developmental processes to create novel morphologies or to conserve similar structures in a variety of organisms. This relatively young field of biology addresses the origin and evolution of body plans and how developmental modifications produce new features.

The field of evo-devo is focused on how regulatory changes or new expression patterns to give rise to new phenotypes. What differentiates a bat wing from the human arm? What new morphologies allowed life in the seas to adapt to terrestrial habitats? Do all animals see their surroundings as we do? Evolutionary developmental biologists are quickly finding that the answers to these questions are not as simple as once thought. The following work summarizes some of the recent efforts in evo-devo to understand these fundamental puzzles and provides insight into future avenues that biologists will be exploring in the coming decade.

Innovation or Elaboration: Debate Over Autopod Origins

Fossil evidence indicates that the tetrapod, or four-limbed vertebrate, evolved during the Devonian era. The origin of the tetrapod limb structure is considered a pivotal event that ultimately allowed for terrestrial exploration and habitation. This fin-to-limb transition gave rise to the autopod, distinct hands and feet in the paired appendages, which greatly enhance terrestrial navigation. The origin of the autopod, however, is a source of much debate, as it is unclear whether the structure arose *de novo* or was derived from a previously established phenotype. According to the *de novo* theory of tetrapod origin, the autopod is an evolutionary novelty that arose independent of the established body plan. Essentially, a

handless organism gave rise to a creature with a fully developed autopod. More likely, however, the autopod is a derived feature that involved the elaboration and differentiation of features already present in fish fins. In this chapter, I present evidence for both perspectives, argue that support for the derivative hypothesis is more compelling, and suggest new avenues for future research.

Derivation

Extensive evidence supporting the notion that the autopod is a derived feature comes from fossilized remains of Devonian fish that represent a morphological and functional transition between fins and limbs. Recently discovered in Nunavut, Canada, the *Tiktaalik roseae* exhibits an array of joints in the distal fin that is functionally similar to the distal limb pattern of early tetrapods.¹ Furthermore, the *Tiktaalik* fin was capable of a range of postures, including a supported stance in

which the shoulder and elbow were flexed in such a way that could have allowed for movement on land. *Gogonasmus*, another Devonian fish discovered in Australia, shows a mosaic of derived tetrapod-like features and shares a number of characteristics with *Tiktaalik*, the most tetrapod-like fish. The cranial structure, for example, displays a number of natural gaps that could have allowed *Gogonasmus* to breathe on land. These spiracular openings may be a precursor to tetrapod middle ear architecture, moving *Gogonasmus* crownward on the phylogenetic tree, closer to basal tetrapods.² The pectoral fin also shares several features with *Tiktaalik*, including a radius and ulna that are offset at similar angles and an unusually short ventral extension. Taken together, these fossils indicate that the autopod may have evolved as an extension of previously established structures in tetrapod-like fish.

In addition to fossil evidence, analysis of *Hox* gene clusters has been used to indicate that a temporal shift in expression patterns, rather than *de novo* activation of *Hox* genes, may have led to the evolution of autopod digits. *Hox* genes are a subset of the homeobox domain sequence that are involved in patterning the major body axes and, subsequently, determine the relative positioning of limbs. During tetrapod limb development, *HoxD* genes are expressed in two phases; initial expression of *Hoxd9* through *Hoxd13* patterns the limb axes while a subsequent wave of transcription regulates digit formation.³ In non-tetrapods, such as zebrafish, the second phase of *Hox* expression does not occur, indicating that the origin of digits in early tetrapods was driven by the addition of a new, distal domain of *Hox* expression.⁴ Recent studies have examined *HoxD* gene expression patterns in the shark *Scyliorhinus canicula*, an early jawed vertebrate that is not a member of the tetrapod family, to

determine whether this second phase of transcription is a novel feature exclusive to tetrapods. While the initial wave of *Hox* expression is consistent with zebrafish and tetrapod patterns, a second phase occurs during the later stages of fin development in which *Hoxd12* and *Hoxd13* are re-expressed in fin buds.⁵ These results indicate that the second phase is not unique to tetrapod digit development and that perhaps a temporal extension of *Hox* expression, rather than novel activation, led to autopod digit evolution.

De Novo

Compelling evidence for the *de novo* viewpoint comes from a different perspective on the biphasic expression of *HoxD* mentioned above. In tetrapods, the late phase of *HoxD* expression extends across the distal portion of the limb that will form the autopod. Zebrafish, however, are missing this late phase and, as a result, lack distal skeletal structures resembling digits. It has been hypothesized, therefore, that this biphasic *HoxD* expression is a tetrapod novelty correlated with evolution of the autopod. Recently, a DNA regulatory element dubbed the “digit enhancer” has been postulated in mice to control the late-phase autopodial *HoxD* expression.⁶ This digit enhancer element has not yet been found in zebrafish, lending support for the hypothesis that digit specification arose *de novo* via a new regulatory element that altered the timing and expression pattern of *Hox* genes.

Further support for this view comes from genetic analyses in which the genes responsible for patterning the anterior-posterior axis in tetrapods have been knocked out. A gradient of *Sonic hedgehog* (*Shh*) signaling is required to maintain the apical ectodermal ridge, a structure overlying and inducing the developing limb bud. Previously, deletion of *HoxA* and *HoxD* clusters in developing mouse forelimbs has been shown to abolish *Shh* expression, resulting in the loss of forearms and digits.⁷ Recently, however, addition of *Hoxd13* alone induced the formation of both digits and a truncated forearm. Tarchini, Duboule, and Kmita interpret this finding to mean that the novel

recruitment of the *Hox* system from the developing trunk into growing limb buds led to an increased number of bony elements along the anterior-posterior axis and their simultaneous patterning. The resulting heteromeric distal appendages likely allowed for the architectural versatility and associated functional feature that accompanied the origin of tetrapods.⁸

There is also evidence that the proximal limb bud and the autopod maintain different molecular mechanisms for chondrification, or cartilage formation. Activin A is a growth factor that plays a role in chondrogenesis during digit formation but can be inhibited by the protein follistatin.⁹ It cannot, however, induce ectopic cartilage formation in the early stages of limb development. Furthermore, follistatin can inhibit cartilage formation only in the autopod. It is ineffective in inhibiting chondrogenesis in the limb bud. Chondrogenesis and, by extension, tetrapod limb development, proceeds through a different molecular pathway in the autopod than in the proximal limb buds.¹⁰

Recent Advancements

The apical ectodermal ridge (AER) is a transient embryonic structure essential for the induction, patterning, and outgrowth of the tetrapod limb.¹¹ Until recently, however, the mechanism of AER function in skeletal patterning has remained unclear. The Progress Zone (PZ) model suggests that the AER provides permissive signals and positional information to keep cells labile until they exit the zone and become specified.¹² Alternatively, the Early Specification (ES) model postulates that proximal-distal information is specified at the earliest stages of limb bud development and that the AER regulates subsequent expansion by promoting cell proliferation.¹³

Although fundamentally different, both the Progress Zone and Early

Specification models imply the existence of a limb bud distal zone where cells remain until they reach the time to differentiate (PZ model) or to expand (ES model). Susana Pascoal suggests a method for how these cells measure time by providing evidence of a molecular clock operating during chick forelimb autopod growth. *Hairy2* is a gene encoding a transcriptional repressor that has recently been implicated in molecular clock machinery.¹⁴ Pascoal analyzed *hairy2* expression during forelimb development and found that, although forelimbs of the same embryo always presented the same expression pattern, forelimbs of different embryos at the same stage of development showed variable *hairy2* patterns. *In situ* hybridization studies revealed that expression peaks after six and twelve hours of incubation, indicating that *hairy2* expression in autopod precursor cells cycles with a 6-hour periodicity. These oscillations are registered by the progenitor cells, providing them a temporal value that could either be translated into positional information (PZ model) or determine the time of expansion (ES model). Although this proposal does not allow for discrimination between the two models, it does introduce the possibility that a molecular clock can be operating in embryonic tissues with tissue-specific time periods, controlling the formation of various morphological units.

Initiation of the AER involves interactions of several major signaling pathways and growth factors. *Fgfr2* is a critical growth factor receptor involved in both limb ectoderm and mesenchyme during development. Mouse embryos lacking *Fgfr2* fail to develop beyond implantation, while those with partial loss of *Fgfr2* function survive to later stages but ultimately fail to develop limbs.¹⁵ In 2008, Lu *et al.* expanded on these findings by genetically ablating the AER through conditional removal of *Fgfr2* function.¹⁶ Premature loss of the apical ectodermal ridge in mutant limb buds delayed generation of autopod progenitors, which in turn failed to reach a threshold number required to form a normal autopod. Interestingly, neither cell survival nor cell proliferation was affected in the distal mesenchyme of *Fgfr2* knockout forelimb

buds. This is in contrast to previous models holding that the AER regulates limb skeletal progenitors by promoting cell survival and proliferation. Rather, this novel mechanism suggests that the apical ectodermal ridge regulates the number of autopod progenitors by determining the onset of their generation.

New Directions

In mouse forelimb buds, sustained growth factor signaling in the distal limb bud mesenchyme results in the generation of autopod progenitors at the 31-32s stage (Figure 1A). The progenitor pool subsequently expands and a sufficient number of skeletal progenitors are available to form a normal autopod when condensation starts at the 46s stage. In forelimb buds lacking *Fgfr2* (Figure 1B), the AER is not maintained because of increased cell death, and growth factor production progressively decreases. FGF signaling in the distal mesenchyme is reduced and generation of autopod progenitors is delayed by two somite stages. Although the progenitor pool expands normally at later stages, it fails to produce a sufficient number of skeletal progenitors to form a normal autopod at the onset of condensation.

It is unknown whether distal *HoxD* domain expansion during the fin-limb transition occurred via modulation of existing regulatory elements, enhancer sequence evolution, or sustained mitogenic factor production. Expansion of the primitive distal *HoxD* domain by sustained signaling from the apical ectodermal ridge is consistent with the notion that the extent of skeletal development is controlled by the timing of the transition of the AER to the apical ectodermal fold (AEF), within which dermal fin rays differentiate. Delaying this transition would result in extended AER signaling and, subsequently, produce a more elaborate endoskeleton. Artificially increasing the amount or signaling period of

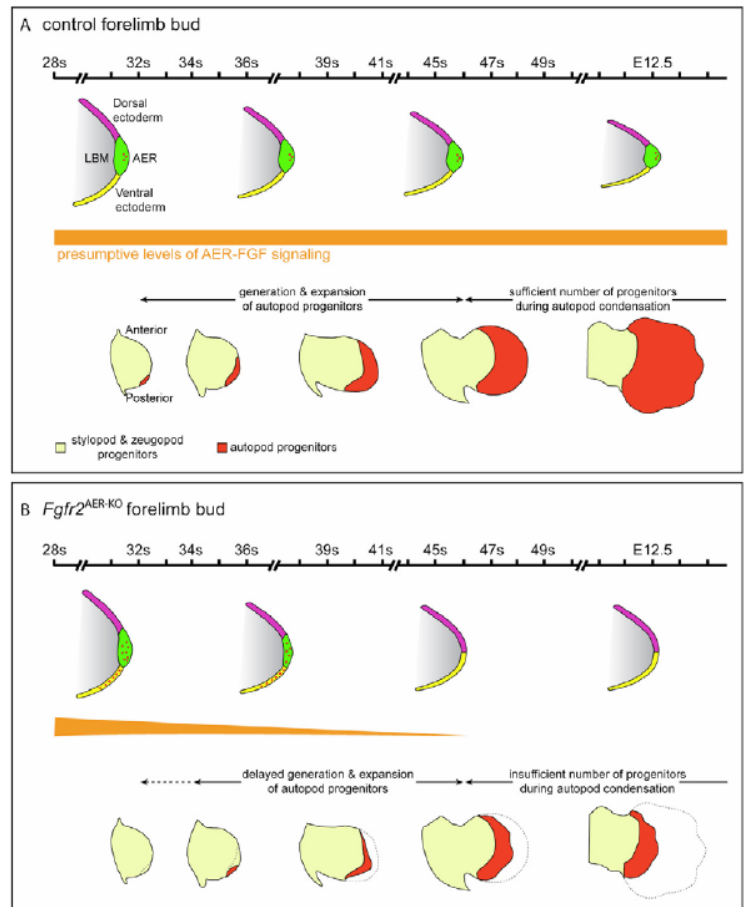


Figure 1: AER function in development of the mouse forelimb autopod. AER maintenance is essential for generation of autopod progenitors. (Lu, 2008)

mitogenic factors or *Fgfr2* would result in sustained expression of *Hoxd13* and cell proliferation, elaborating the distal limb skeleton. *In situ* hybridization assays for *Hox* expression should produce results similar to those in Figure 1A, with increased numbers of autopod progenitors and, consequently, a more elaborate skeletal structure. Thus, a temporal extension, rather than *de novo* activation, of *Hox* expression in the distal fin may have led to digit development during the fin-to-limb transition.

A promising avenue for future research addressing the question of whether modulation of existing regulatory elements is responsible for this shift in *Hox* expression explores microRNA (miRNA) function. MicroRNAs are small, single-stranded RNA molecules capable of binding to complementary sequences in mRNA molecules.

Formed from longer RNA precursors that fold back on themselves and are cleaved into single strands by a Dicer enzyme, miRNAs form a complex with one or more proteins. This miRNA-protein complex then either degrades the target mRNA or blocks its translation. This endogenous interference with gene expression is similar to RNA interference (RNAi), an experimental technique in which the injection of double-stranded RNA molecules into a cell turns off expression of genes with similar RNA sequences.

It has been estimated that expression of nearly one third of all human genes may be regulated by miRNAs.¹⁷ Given this figure, it is likely that miRNAs could serve as global enhancers of distal *Hox* gene expression. Using northern analyses, quantitative PCR, or microarrays, nucleic acid probes could be designed to search for miRNAs that affect *Hox* mRNA translation. Once found, these miRNAs could be ectopically expressed at various times during autopod development. *In situ* hybridizations could be used to observe their effects on distal *Hox* expression, the timing of the AER transition, and progression of autopod development. The evolution of a single microRNA would lend support to the *de novo* hypothesis, as changes in *Hox* gene expression would result from a novel regulatory element.

Summary

Ultimately, whether the autopod arose *de novo* or was derived from an established anatomical structure is a debate best reserved for semantics. Phenotypically, the tetrapod limb is an evolutionary innovation because the autopod developed independent of the previous body plan. While these changes may have arisen *de novo* via mutation, the new sequences generated are derived from established pathways. Clearly, the autopod is a structure

exclusive to tetrapods that originated through a number of mechanisms or regulatory changes. In this way, it is derived from structures in a series of intermediate, tetrapod-like species throughout the Devonian era. This transition from fish fins to paired appendages in tetrapods was a critical step toward making life on land possible for many organisms.

Overcoming Developmental Constraints:

Evolution of the Vertebrate Jaw

It is generally believed that the jaw is one of the earliest innovations in vertebrate evolution. During development of jawed vertebrates, or gnathostomes, cranial neural crest cells migrate into the pharyngeal arches (PAs) and ultimately form the lower jaw. A similar migration occurs during agnathan (jawless vertebrate) development, yet the first pharyngeal arch necessary for jaw formation does not arise. It has been proposed that two critical events must have occurred in order for jaws to evolve and allow for terrestrial habitation. First, a change in *Hox* gene expression dictated a new set of instructions for pharyngeal arch development. Second, a shift in the timing of germ layer interactions established a permissive environment allowing for novel cell migratory patterns. Here, I summarize theories regarding these heterotopic and heterochronic shifts that set the stage for jaw evolution and offer suggestions for new avenues of research.

New Instructions

The head of the vertebrate embryo is characterized by possession of dorsoventrally articulated jaws derived from the rostral-most pharyngeal (mandibular) arch. While the evolution of the jaw has traditionally been viewed as the establishment of a developmental program for the ectomesenchyme of this mandibular arch (MA) to form the upper and lower jaws, the history of this change remains unknown.¹⁸ In the gnathostome embryo, a specific class of homeobox genes are expressed along the anteroposterior axis of the pharynx. These *Hox* genes are arranged in clusters,

so that each pharyngeal arch carries a unique set of transcripts. This ‘*Hox* code’ provides positional information and establishes the boundaries of the pharyngeal arches. Only the mandibular arch lacks expression of *Hox* transcripts, and for this reason the first pharyngeal arch is described as the ‘*Hox*-free default state.’

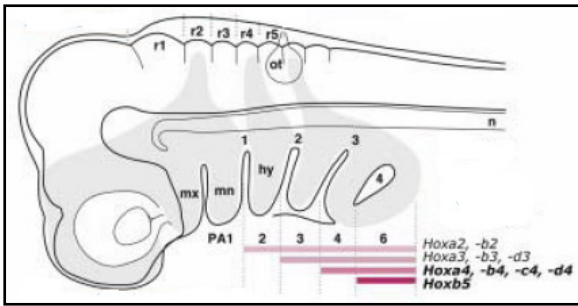


Figure 2: Gnathostome gene expression patterns. *Hox* transcripts are distributed in a nested pattern in the PAs with the MA defined by the *Hox*-free state. (Adapted from Kuratani, 2004)

the mandibular arch governs gnathostome jaw patterning. Although *Hoxa-2* is normally restricted to the hyoid arch (PA2) and posterior, ectopically expressing this gene in the MA transforms it into the hyoid arch. Similarly, removing *Hox* expression from PA2 results in transformation of that tissue into MA.¹⁹ Furthermore, transplantation of *Hox*-free neural crest cells into tissue that is destined to become the hyoid arch results in the duplication of MA skeletal elements.²⁰ The differentiation of the jaw, therefore, is permitted by the absence of *Hox* gene expression from the mandibular arch.

Permissive Environment

The only extant groups of agnathans are the lampreys and hagfish and, of these, only the lamprey is used for embryological comparisons with gnathostomes. Although the early embryonic pattern of the lamprey is similar to that of jawed vertebrates, distinct migratory patterns of neural crest

cells during development give rise to strikingly different oral apparatuses.²¹ The gnathostome mouth consists of the upper and lower jaws, while the upper and lower lips of the lamprey form its funnel-like oral apparatus. Agnathans form a naso-hypophyseal plate during development from which the nasal epithelium and pituitary arise. This epithelial plate forms a barrier to neural crest cell migration that redirects the cells underneath the plate. Reaching their destination, these crest cells form the upper lip of the lamprey mouth. In jawed vertebrates, the naso-hypophyseal plate separates into the nasal placode and Rathke’s pouch, allowing space through which the crest cells can migrate to form the lower jaw.²²

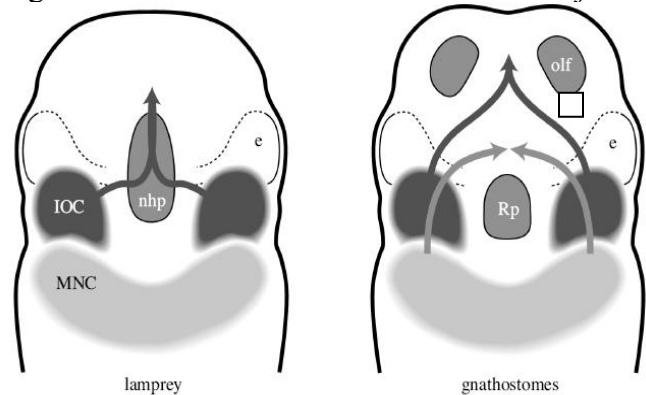


Figure 3: Comparison between lamprey and gnathostome embryos. In the lamprey, the upper lip develops from crest cells (IOC) that migrate rostrally beneath the naso-hypophyseal plate (nhp). In gnathostomes, cells grow rostrally between Rathke’s pouch (Rp) and the olfactory placode (olf).

The difference in migration allowing formation of the jaw may be a product of heterochronic shift. Changes in the timing of embryonic events often cause shifts of cell and tissue relationships. If separation of the nasal placode and pituitary occurs early in development, jaws are possible. If the separation is late, a barrier prevents crest cell migration into the region that would otherwise give rise to the mandible.²³ Removal of this barrier via a heterochronic shift during development established the permissive environment necessary for the evolution of the vertebrate jaw.

Recent Advancements

In 2005, Shigetani, Sugahara, and Kuratani proposed a new evolutionary scenario for jaw patterning. In their interpretation, jaw evolution involved a topographical shift of growth factor distribution in the ventral ectoderm. This heterotopy disrupts the relationship of homologous structures, bringing about a new development pattern. The expression domains of growth factors *Dlx* and *Msx* were always associated with the proximal and distal parts of oral protrusions in both the lamprey and gnathostomes, respectively.²⁴ Regardless of the morphological identities of the ectomesenchyme, the regulatory system of these genes may have been utilized for the jaws and lips.

Because there are patterns that are unchanged between lampreys and gnathostomes, it can be assumed that these developmental patterns were already present in the common vertebrate ancestor. More specifically, the hypothetical ancestor possessed neural-crest-derived ectomesenchyme distributed in the ventral head in a pattern similar to extant vertebrate embryos (Figure 4A). The visceral cartilage was patterned through signals from a similar embryonic environment at each level. A *Hox* code like that of gnathostomes existed along the AP axis of the pharynx, while the rostral ectomesenchyme was devoid of *Hox* transcripts. This ectomesenchyme differentiated into an oral apparatus with the aid of homeobox genes.

Both in lampreys and in gnathostomes, the growth factor *Fgf8* is associated with the proximal part of the oral apparatus, and the growth factor *Bmp4* is associated with the distal parts. Although the function of these genes in shaping the apparatus is conserved, the morphological identities of the ectomesenchyme may not

be shared. A heterotopic, caudal shift of the pharyngeal FGF8/BMP4 expression defined the new oral region in gnathostomes, while the ectomesenchyme that used to differentiate as the agnathan upper lip came to form the gnathostome

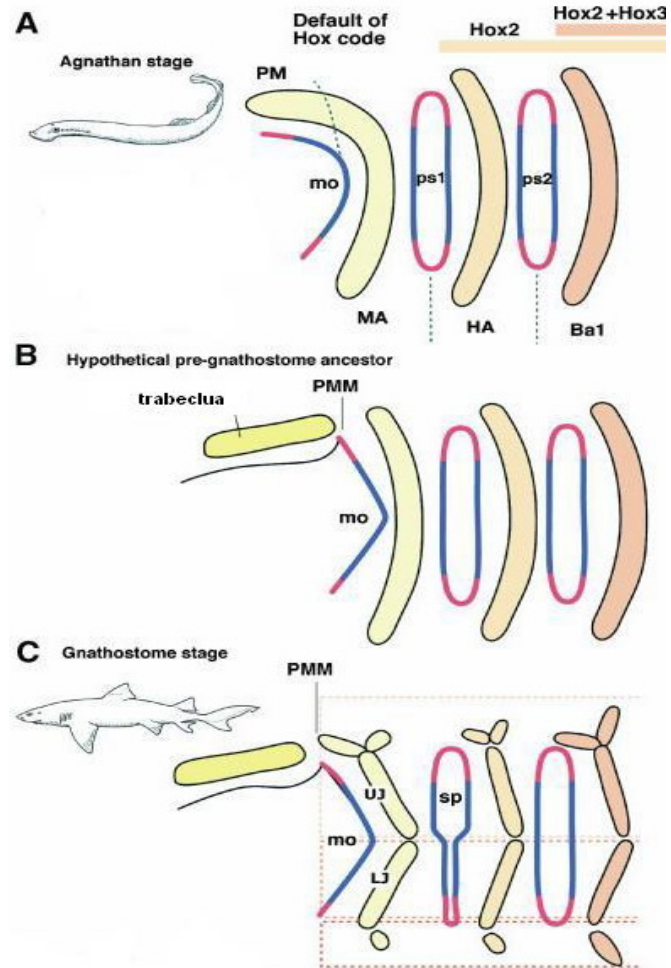


Figure 4: Hypothetical scenario of jaw evolution. A: Head ectomesenchyme is divided into oral regions (PA & MA) and visceral arches (HA, Ba1). Both the dorsal and ventral oral protrusions are differentiated through cascades involving FGF8 (blue) and BMP4 (red). B: More *Hox* genes have acquired collinear expression patterns in the pharyngeal arches. Due to the heterotopic caudal shift of growth factors, the anterior ectomesenchyme is divided into PM and MA. The PM ectomesenchyme is now free from an oral apparatus shaping program, to be incorporated into the trabecula. C: The gnathostome-type basic structure has been established. (Adapted from Shigetani, 2005)

trabecula (Figure 4B). In addition to the redefinition of the oral region, a DV specification is brought about in arch skeletons (Figure 4C). This specification can be recognized as the establishment of the gnathostome version of the default skeletal pattern.

New Directions

It is unknown whether the *Hox*-free state of the MA was established at the outset of gnathostome evolution, or if it was already present in agnathans or invertebrates. A preliminary study on lamprey *HoxL6* reports that the gene is developmentally upregulated throughout the pharyngeal arches, indicating that *Hox* transcripts in agnathans inhibited jaw differentiation.²⁵ A second study, however, contradicts this conclusion. Takio et al. isolated 11 *Hox* genes from a different species of lamprey, including the *HoxL6* ortholog, but none were expressed in the embryonic mandibular arch.²⁶ Nearly all vertebrates share differentiated mandibular and hyoid arches, and it is therefore likely that the *Hox* code was at least established in the lamprey-gnathostome common ancestor. This suggests that the developmental program that gives rise to the jaw involves changes in molecular mechanisms downstream of the shared *Hox* code.

Examining the regulatory pathways and expression patterns of toolkit genes responsible for axis patterning, like *Dlx* or *Otx*, may shed light on why the absence of *Hox* transcripts is necessary for the permissive environment. *Dlx* genes, for example, are expressed in the forebrain, cephalic neural crest, otic vesicle, olfactory placodes, pharyngeal arches, limb buds, and teeth.²⁷ These sites of *Dlx* expression are major morphological innovations along the vertebrate lineage. Comparing the number of *Dlx* genes (six families in gnathostomes vs. a single *Dlx* ortholog in lampreys) and their

phylogenetic relationships allowed Neidert et al. to suggest that a tandem duplication of an ancestral *Dlx* gene predated the divergence of lampreys and gnathostomes. Similar efforts with *Otx* involving cloning of *Otx* homologs in lamprey, nucleotide sequence analysis, and *in situ* hybridizations would provide insight into the evolution of the *Otx* gene family and its function in morphological innovation.

Additionally, it is important to note that molecules involved in patterning of the gnathostome jaw are used in similar patterning of the upper and lower lips in agnathans. Namely, the growth factor *Fgf8* is widely expressed in the lips and jaws while *Bmp4* is expressed in the lamprey oral apparatus and gnathostome mouth. Homologous sets of genes, therefore, are not expressed in homologous embryonic regions, but rather in structures that are functionally homologous. It would be interesting to express these growth factors ectopically or implant beads into different embryonic tissue and observe whether the resulting morphology is affected. For example, implantation of a lamprey *Fgf8* cognate bead into the gnathostome premandibular region may result in lamprey-like morphology, while mimicking gnathostome *Fgf8* or *Bmp4* expression in the lamprey may result in rudimentary jaw characteristics. This causative experiment may indicate that a topographic shift in epithelial-mesenchymal interactions occurred during the transition from jawless to jawed states.

Furthermore, only a small number of regulatory genes involved in oral patterning have been examined. A systematic search of genes expressed in the mandibular arch using techniques like DNA microarrays will undoubtedly reveal more molecules to study. These studies will be able to describe changes in the genetic cascade, regulation of genes, and new developmental patterns by establishing a conceptual framework with which to examine morphological homology and changes in developmental constraints.

Summary

The vertebrate jaw is an evolutionary novelty brought about by overriding ancestral developmental constraints. Through the removal of a physical barrier and the establishment of the *Hox*-free default state, a new phenotype arose that is no longer homologous with the ancestral pattern. Although the vertebrate mouth and agnathan oral apparatus maintain similar functions, their developmental origins have been altered. The significance of this novelty cannot be understated, as life in terrestrial habitats would simply not be possible without the jaw. Furthermore, the possibility that homologous structures may not be patterned by equivalent cell populations has implications for other areas of evolutionary developmental biology and strengthens the notion that the regulation of genetic pathways, rather than changes in pathway components themselves, is the leading cause of morphological variation.

Evolutionary Foresight: Envisioning The Eye

Even Charles Darwin had to concede that it seems “absurd in the highest possible degree²⁸” to suggest that the eye, with its ability to admit different amounts of light, adjust for aberrations, and focus to various distances, could have evolved by natural selection. Indeed, the eye is an organ of such complexity that it challenges both evolutionary biologists attempting to explain its phylogenetic origins and developmental biologists trying to describe its formation during ontogeny. Since the discovery that the transcription factor *Pax6* plays a critical role in specifying the eye, there have been two main points of contention among biologists with regards to the evolutionary origins of the eye. First, although *Pax6* has been shown to play a key role in eye development throughout the animal kingdom, doubt remains as to whether it can

be described as a “master regulator” gene.²⁹ Second, while morphological comparisons of eye anatomy and photoreceptor cells led to the view that animal eyes arose multiple times independently, the molecular conservation of *Pax6* has indicated the opposite – that the eye evolved from a single precursor and diversified by adaptive radiation.³⁰ Here, I summarize evidence regarding both debates and suggest new directions for future research.

Master Control Gene

Walter Gehring first described the central role of *Pax6* in eye development, observing that mutations in the gene disrupt normal progression of the eye. Employing gain-of-function mutations of both *eyeless* (the first *Drosophila* mutation discovered) and mouse *Pax6*, Gehring induced ectopic growth of complete compound eyes on the antennae, wings, and legs of the fly.³¹ Furthermore, the mouse *Pax6* gene is capable of inducing ectopic eyes in the *Drosophila*, showing that *Pax6* homologs of insects and mammals serve homologous functions. Because this gene sits on top of the genetic hierarchy shared by insects and mammals, it has been considered a “master control gene” capable of regulating the entire pathway of eye development.

Recent evidence, however, has called this theory into question. The jellyfish *Tripedalia* has a *PaxB* gene that appears to be a hybrid between *Pax6* and *Pax2/5/8*. This *PaxB* gene is capable of inducing ectopic eyes in *Drosophila* and rescuing *Pax2* mutants.³² The *Tripedalia* eye also serves as a balancing organ, suggesting that a Bilaterian duplication of the *PaxB* gene may have allowed *Pax6* to specialize for regulation of eye development while *Pax2/5/8* evolved to control the ear. Thus, while the ancestral *PaxB* was responsible for eye development in cnidarians, *Pax6* arose from a common ancestor with *PaxB* only after the separation of Cnidaria from Bilateria. Although *Pax6* plays a critical role in eye formation throughout the animal kingdom, it is clearly not required for proper development in every species.

Multiplicity

The determination of whether *Pax6* is indeed a master regulator of eye development is critical to understanding the evolutionary origin of the structure. Classical morphological studies have pointed to the distinct ontogenetic origins of eyes in different species as evidence that the eye has evolved independently in at least 40 phyla.³³ The vertebrate retina, for example, arises from neural ectoderm and induces head ectoderm to form the lens, while cephalopod retinas result from invaginations of the lateral head ectoderm, producing an eye that lacks a cornea.³⁴ Furthermore, a simulation model selecting for improved visual acuity produced a camera-type eye in less than 4×10^5 generations (a half million years).³⁵ If the eye has evolved independently multiple times, it seems unlikely that *Pax6* alone could be responsible for each occurrence.

Gehring, however, challenges these ideas and argues for a monophyletic origin of the eyes from precambrian ancestors in which the diversity of eye types observed today arose via adaptive radiation.³⁶ He first points to *sine oculis* (*so*) as another master control gene of eye development, citing its ability to regulate *eyeless* and rescue null mutants.³⁷ Because both *Pax6* and *sine oculis* are present in the primitive eyes of flatworms, Gehring proposes that more elaborate eyes have evolved by intercalation or recruitment of additional genes into the eye developmental pathway. *Eyeless* (*ey*), for example, has been intercalated into the eye pathway between *twin of eyeless* (*toy*) and *sine oculis*. Since *toy* and *ey* both interact with the same enhancer inside *so*, it suggests that they are derived from a common ancestral gene. This is in agreement with the similarity of their amino acid sequences and the fact that they can partially substitute each other. Gehring

then points to *Drosocrystallin* as an example of a crystalline gene in vertebrates that has been recruited into the lens developmental pathway from another source.³⁸ *Drosocrystallin* belongs to the family of cuticle proteins, suggesting that it was recruited into the eye pathway by fusing to a lens-specific enhancer. While differential expression of conserved genes and recruitment of genes into the eye morphogenetic pathway provide a mechanism for the evolution of different eye types originating from the same prototype, Gehring's argument is reliant upon *Pax6* and *sine oculis* serving as master control genes – a stance which has not yet been verified.

Recent Advancements

Biosynthetic pathways are generally linear, with many enzymatic steps leading from the original substrates to a final biosynthetic product. This linearity poses a problem when considering how such pathways could evolve in organisms lacking the appropriate enzymes. Proposed by Horowitz in 1945, a retrograde mode of evolution solves this problem by introducing an organism that is unable to synthesize the desired product and must take it up from the environment.³⁹ When the supply in the environment was exhausted, those organisms that had the final enzyme in the pathway could make use of the immediate precursor, until the supply of precursor was also exhausted. Then, only those organisms possessing the next enzyme could survive, and so on until the biosynthetic pathway was established.

For the evolution of morphogenetic pathways, like eye morphogenesis, Gehring proposes a mechanism of intercalary evolution.⁴⁰ The eye prototype, which is the result of a purely random event that assembles a photoreceptor and pigment cell into a visual organ, requires the function of at least two gene classes: a master control gene, *Pax6*, and the structural genes encoding the genetic cascade. Starting from this prototype, more sophisticated eye types arose by recruiting additional genes into the morphogenetic pathway. At least two mechanisms, gene duplication and enhancer fusion, lead to the

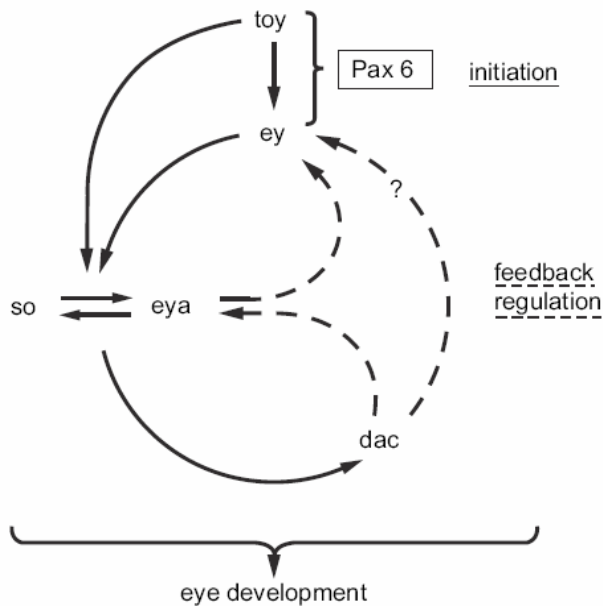


Figure 5: Gene regulatory network controlling eye determination (Gehring, 2005)

intercalation of additional genes into such a cascade.

A case of gene duplication can be illustrated with *eyeless* and *twin of eyeless*. Primitive insects possess a single *Pax6* gene, whereas *Drosophila ey* and *toy* genes have functionally diverged in evolution. The positive autocatalytic feedback loop found in *Pax6* has evolved into a heterocatalytic loop by which *toy* activates *ey*, leading to the intercalation of *ey* underneath *toy* into the morphogenetic pathway (Figure 5). Enhancer fusion can be illustrated by the *Drosophila* lens protein drosocrystallin, which is not conserved in evolution and found only in insects.⁴¹ By fusing it to a lens-specific enhancer, Gehring recruited *Drosocrystallin*, a cuticle protein, into the eye development pathway. However, because this evolution of a prototypic eye is such a highly improbable event that is not driven by selection, Gehring maintains that the hypothesis of a polyphyletic origin of the eyes is unlikely.

In 2007, Zbynek Kozmik established a bipartite model to provide a plausible

explanation for the apparently ancient role on *Pax* genes in eye evolution (Figure 6).⁴² These genes encode transcription factors defined by the presence of a highly conserved DNA binding paired domain (PD). In addition to PD, some Pax proteins, including Pax6, contain a homeodomain (HD) that interacts with palindromic target sequences. Because these two independent binding domains can operate individually or cooperatively, Pax proteins regulate an unusually broad spectrum of target genes. Kozmik's bipartite model suggests that this unique nature of Pax transcription factors was the key determinant for selecting them to form the prototypical eye structure. Specifically, the two DNA binding domains within a single Pax transcription factor have been co-opted for two essential features of the prototypical eye. PD-regulated genes control production of a dark shielding pigment, while HD controls production of a photopigment. Once this transcriptional regulation was established, the two genetic programs being driven by two independent DNA binding domains within a single transcription factor became inseparable.

New Directions

Ironically, clues about *Pax6* and its role in the evolutionary origins of the eye may come from studies involving the loss of sight in cave fish. The mechanisms responsible for eye degeneration in cave-adapted fish have not yet been resolved, but two competing theories have emerged. The neutral mutation hypothesis suggests that eye regression is caused by random mutations in eye-forming genes which accumulate under relaxed selective pressure,⁴³ while the adaptation hypothesis states that loss of eyes is adaptive for a cave environment in which sensory organs beneficial to survival are enhanced at the expense of eyes.⁴⁴ Recent evidence, however, seems to contradict both hypotheses. In blind cavefish, at least the majority of genes responsible for normal eye development are initially expressed.⁴⁵ Furthermore, it seems that formation of a rudimentary eye is necessary, despite its energetic cost, because of a requirement

for the normal development of other facial structures.⁴⁶

During normal eye development, *Hedgehog* signaling inhibits *Pax6* expression in the midline to create two lateral eyes. In 2005, William Jeffery injected *Hh* mRNA into one side of a surface fish egg and found that, later in development, *Pax6* expression was downregulated and resulted in a smaller optic vesicle, smaller eye, and an optic cup that lacked a ventral section. He hypothesized that the molecular chaperone *hsp90α*, a possible downstream target of *Hh* signaling, is responsible for lens apoptosis and eye degeneration. Furthermore, comparisons of *Pax6* expression between cavefish and surface-dwelling species found that the gene is reduced in cavefish, its expression domains are diminished, and the left and right *Pax6*-expressing domains lack a connecting zone spanning the midline (resulting in a gap between the optic vesicle fields). It will be important to understand how and why *Pax6* expression patterns are modified in cavefish and what effect these changes have on possible downstream effectors like *hsp90α*.

To determine if *hsp90α* is a downstream target of *Hh* signaling, it would be useful to compare gene expression patterns under conditions of *Hh* inhibition and over-expression. The TUNEL assay is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades. Isolating *hsp90α* DNA to use as probes for *in situ* studies during surface fish and cavefish development would help elucidate the chaperone's role in apoptosis and examine the timing of *hsp90α* expression. Although this study may not provide insight regarding the origin of the eye, it could reveal information on evolutionary processes like the generation of novel phenotypes.

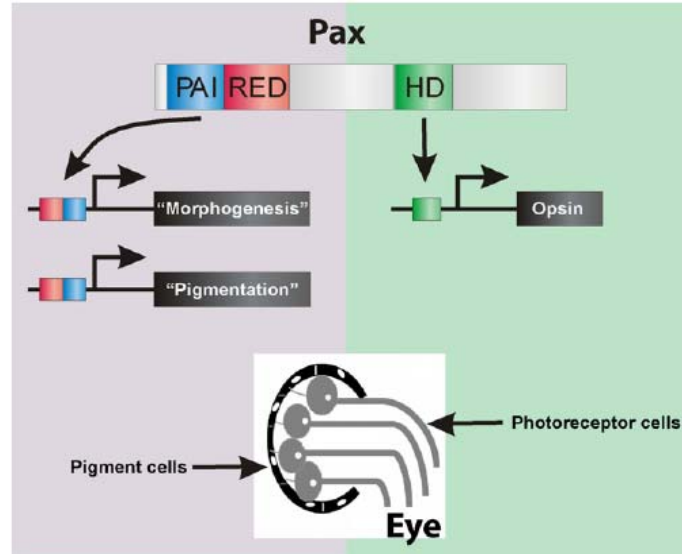


Figure 6: Bipartite model of eye evolution. (Kozmik, 2008)

Cells may prevent apoptosis through a number of mechanisms including mitogens that stimulate cell division, growth factors that promote cell growth, and survival factors that prevent death by suppressing apoptotic mechanisms. Recent efforts have shown that inhibiting *hsp90α* function suppresses lens apoptosis, and it has been hypothesized that the protein normally serves as a chaperone to help activate cell death factors.⁴⁷ It is also possible that, because *hsp90α* can be secreted outside cells, it may have effects throughout the deteriorating cavefish eye. This duality provides an excellent mechanism with which to analyze the function of *hsp90α* in apoptosis and determine whether it acts intracellularly to promote cell death factors or extracellularly to inhibit survival factors. When external factors are required to prevent apoptosis, a cell surface receptor detects the survival factor, triggering an anti-apoptotic signaling cascade that begins with protein kinase B (PKB) and Hid protein in mammals and drosophila, respectively. Culturing cells with varying amounts of survival factor and *hsp90α* protein and comparing survival rates would determine if the chaperone acts extracellularly. It would either compete directly with survival factors for the active site of the receptor or indirectly by

altering the receptor and/or survival factor so that they are no longer compatible. Alternatively, characterization of *hsp90α*'s intracellular activity could be gauged through selective knockout experiments. Abolishing the function of each of the proteins involved in the apoptotic cascades like PKB, Hid, caspases, Bad, or Bcl-2, will allow determination of which event in the signal transduction sequence *hsp90α* acts upon.

Summary

The formation of the eye remains an intriguing topic in evolutionary

developmental biology because it represents a novel mechanism rarely, if ever, seen in genetics. The possibility of a single gene sitting atop a cascade of downstream effectors, governing the formation of an entire organ as a “master regulator” of expression poses interesting questions concerning every other developmental pathway so far discovered. Just as Darwin struggled to explain how the complexity and seeming perfection of the eye could have arisen via natural selection, so too are today's biologists only beginning to understand the intricacies of the regulatory pathways that ultimately build the human body.

Afterword

Evolutionary developmental biology is a versatile tool that provides explanations for some of the central questions of evolutionary theory. Through mechanisms like heterotopy and heterochrony, Evo-Devo describes how novel morphologies arise and are maintained, how the major body axes are patterned, and how these elements interact to drive the evolution of new species. *Hox* genes, conserved amongst numerous species, not only support the idea of a common origin of all life on Earth, but also suggest a mechanism for the evolution and diversification of body plans. The timing, amount, and location of gene expression are just a few factors contributing to the diversity of life on Earth. Scientists have just begun examining what makes each species unique. These apparent differences, although once seen as innumerable, are quickly being reduced through compelling genetic comparisons, field studies, and cutting-edge experiments.

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