Int. J. Dev. Biol. 44: 29-34 (2000)

Embryonic development of heads, skeletons and amphioxus: Edwin S. Goodrich revisited

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Edwin Stephen Goodrich was one of the greatest zoologists that Britain has ever produced (Fig. 1). Born in 1868, he spent most of his scientific career at the University of Oxford where he held the Linacre Chair of Zoology from 1921 until shortly before his death in 1946. Goodrich's zoological discoveries, descriptions and theories included contributions to palaeontology, systematics, vertebrate anatomy, invertebrate anatomy, physiology and the nature of homology (Hardy, 1947). Remarkable diversity of interests was certainly one of Goodrich's strengths, but it was not achieved at the expense of rigour or depth. On the contrary, the fields of evolutionary biology and comparative anatomy owe a great debt to the contributions of E.S. Goodrich. But what of developmental biology? Even though his most famous work includes 'development' in its title (Goodrich, 1930), Goodrich is certainly not considered to be a founding figure in developmental biology. Despite the fact that he published relatively few papers about embryos, several of Goodrich's observations or hypotheses drawn from other fields have provided an important framework for understanding some recent findings in molecular developmental biology. Here, I revisit some of Goodrich's findings in relation to recent research in developmental biology, focusing specifically on three subjects: the nature of amphioxus, segmentation of the vertebrate head, and patterning of the vertebrate skeleton. In the first two cases, research in my laboratory has helped to test hypotheses proposed by Goodrich; in the third case, others have added mechanistic detail to the theoretical framework he laid down.

The nature of amphioxus

The phylum of animals to which we belong, the Chordata, is usually divided into three subphyla: the Vertebrata (used here as equivalent to Craniata), the Cephalochordata and the Urochordata. These three groups share a common ancestor not shared with any other living animals, and can be considered closely related. The Cephalochordata contains just over twenty species of marine invertebrates known as lancelets or amphioxus. These animals share several prominent features with vertebrates, including the presence of a notochord, dorsal hollow nerve cord, segmented muscle blocks and gill slits opening from the pharynx. They differ from vertebrates in lacking a pronounced head region, neural crest cells, paraxial skeletal tissue and some of the complex visceral organs. A key question that has been asked repeatedly is whether the amphioxus body plan is derived from a precursor of vertebrates (retaining features of vertebrate ancestors) or whether it evolved from a fully fledged vertebrate (by loss of certain features). In short, is amphioxus primitive or degenerate?

The view that amphioxus was degenerate from a more complex fish-like animal had many champions in the second half of the nineteenth century, although the view also had its dissenters. The proceedings of the discussion meeting on the origin of vertebrates held at the Linnean Society of London in 1910 makes absorbing (and amusing) reading, and summarize the competing views held

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0214-6282/2000/$20.00

Printed in Spain

Fig. 1. Edwin Stephen Goodrich. *Photograph courtesy of the Department of Zoology, University of Oxford.*

at that time (Gaskell *et al.*, 1910; Gee, 1996). At this time, Goodrich already had first hand experience of amphioxus from visits to Naples, and had published his initial findings on its excretory system (he was to publish further papers later on amphioxus development). At the Discussion meeting in 1910, Goodrich argued lucidly that amphioxus was a primitive chordate, and not a degenerate vertebrate. He admitted that, "amphioxus is doubtless in some respects a very specialized animal", but he went on to stress that, "it preserves many primitive characters". His assessment of the various characters lead him to argue that "it cannot seriously be urged that it [amphioxus] once possessed in well-developed condition those paired sense-organs which have so profoundly modified the structure of the head region in the Craniata. For it would be ridiculous to suppose that the modified segments could be restored to their original condition of uniformity with the trunk segments; no trace of the disturbance appearing in either adult or embryo."

Around eight years ago, my laboratory set out to investigate the molecular control of developmental patterning in amphioxus. One result of this work has been to confirm Goodrich's view on the primitive nature of amphioxus. The clinching data have come from studies of gene numbers in vertebrates and amphioxus (Holland *et al.*, 1994a,b; Sharman and Holland, 1996). It has become clear that many genes with roles in early developmental patterning of vertebrate embryos exist in small gene families. Most commonly, these have either two members (as in mammalian *En, Otx, Emx, Evx, IGF)*, three members (as in mammalian *hh, Msx, HNF3)*, or four members *(btd/SP, myogenic* genes etc). Only rarely do developmentally expressed genes exist as singletons. Conversely, when gene families appear to contain substantially more than four genes these are obviously divisible into subfamilies. Thus, the 39 mouse *Hox* genes are more accurately viewed as 13 subfamilies (the paralogous groups) containing two, three or four genes; the nine mouse *Pax* genes are divisible into four subfamilies each with two or three genes *(Pax1/Pax9; Pax3/Pax7; Pax2/Pax5/Pax8*; possibly *Pax4* with *Pax6).* This multiplicity of closely related developmentally expressed genes is a feature of all vertebrates examined to date. The same situation is not seen in invertebrates (such as ascidia, echinoderms or nematodes), although *Drosophila* has independently duplicated some developmental genes by a different route.

Amphioxus is one invertebrate in which the complexity of gene families has been studied extensively, both by my laboratory and by others. An inventory of cloned amphioxus genes is given by Holland (1996). The striking observation is that for almost every gene family examined, vertebrates have multiple related genes, whilst amphioxus has just one. For example, amphioxus has a single *Hox* gene cluster (Garcia-Fernández and Holland, 1994), a single *Pax-1/9* homolog (Holland, N.D. *et al.*, 1995), a single *Otx* (Williams and Holland, 1996), a single *En* (Holland L.Z. *et al.*, 1997) and a single *Msx* (Holland *et al.*, 1994a). Such a situation is unlikely to have arisen if amphioxus was degenerate from a vertebrate, as all traces of the vertebrate-specific gene duplications would have had to be purged from the genome. In fact, even if such a highly improbable set of events had occurred, this would still be detectable by molecular phylogenetic analysis of the genes. Such analyses, however, only serve to confirm that amphioxus retains the primitive condition for chordates. A few gene families do not follow the trend, being present in pairs in amphioxus; these include *Brachyury*, myogenic genes and *HNF3* (Holland, P.W.H. *et al.*, 1995; Araki *et al.*, 1996; Shimeld, 1997). Even these cases turn out to favor the 'primitive not derived' view, since molecular phylogenetic analyses show that each pair is resultant from a gene duplication independent of those in vertebrates.

The segmented head

The idea that the vertebrate head is fundamentally segmented dates back at least two hundred years, to when Goethe reputably had a flash of inspiration on finding a dislocated sheep skull in a Venetian cemetery (de Beer, 1937). Unravelling the details of head segmentation has, however, proven difficult. The major problem faced is that the paraxial mesoderm in the cranial region is not divided into clear somites, unlike in the trunk, even at early developmental stages. Histological descriptions of vertebrate embryos by Balfour and others drew attention to mesodermal cavities in the head region that could be serial homologs of somites in the trunk (de Beer, 1937). Goodrich added further data to the picture, principally from histological sectioning of dogfish embryos (Goodrich, 1918). Most importantly, however, he attempted to integrate all the relevant data into a model of vertebrate head segmentation that could be applied to all jawed vertebrates

(Goodrich, 1930). Many others have re-investigated the question since that time but, even today, Goodrich's model remains the principal framework to which comparisons are made (Gilland and Baker, 1993; Northcutt, 1993; Kuratani, 1997).

The key points of Goodrich's model are simple. He argued there are three mesodermal segments anterior to the otic vesicle (named the premandibular, mandibular and hyoid), with probably a further two obliterated by the evolution of the otic capsule (such that they form little or no myotome). Second, he suggested that all cranial nerves, visceral arches and cranial muscles can be assigned to particular segments. Although this model has been influential, it is not universally accepted. Points of debate include whether the head cavities truly mark the remnants of somites (since the surrounding mesoderm does not have intersegmental clefts, and does not produce skeletal tissue), whether the cranial nerves follow a repetitive pattern, whether visceral arches are part of the same segmental series as putative mesodermal segments, and the precise number of segments rostral to and adjacent to the otic vesicle.

A.S. Romer wrote: "The study of segmentation is comparable to the study of the Apocalypse. That way leads to madness" (Thomson, 1993). Despite this warning, several people, including myself, saw the discovery of *Hox* genes in the 1980s as a means to revisit the head segmentation question. *Hox* genes are expressed and functional during embryonic development, but each is only expressed within a characteristic "stripe" or "domain" of the body axis. Since the rostral limit of this domain differs between most *Hox* genes within a species, distinct genes will be active at distinct positions along the axis (Gaunt *et al.*, 1988; Kessel, 1991). These *Hox* gene expression sites are not simply passive reflections of position, rather their gene products actually contribute to specification of position-specific cell fates. Hence, if *Hox* genes could be found that were active in the vertebrate head, and their expression compared with their homologs in the ancestors of vertebrates, the origin of the vertebrate head could be solved. Unfortunately, there is a rather serious problem: vertebrate ancestors, by definition, are extinct. Although the ideal experiment cannot be performed, therefore, we reasoned that amphioxus could act as a suitable proxy. As described in the above section, the cephalochordate amphioxus has retained characteristics of vertebrate ancestors. Furthermore, it possesses *Hox* genes that compare closely (on the basis of DNA sequence) to vertebrate *Hox* genes, including homologs of the most anteriorly expressed paralogous groups (Garcia-Fernández and Holland, 1994). Thus, the amphioxus *AmphiHox-1* gene is closely related to vertebrate genes of paralogy group 1, *AmphiHox-2* is related to paralogy group 2 and *AmphiHox-3* to group 3.

Examination of the embryonic expression of *AmphiHox-1* and *AmphiHox-3* in amphioxus embryos revealed spatially-restricted expression (as expected for *Hox* genes), with the former gene having a more anterior expression limit than the latter (Holland *et al.*, 1992; Holland and Garcia-Fernández, 1996, Wada *et al.*, 1999). Unlike vertebrate *Hox* genes, however, spatial expression of the amphioxus genes seems to be confined to the developing neural tube. Lack of stable expression in amphioxus somites makes comparison to vertebrates more complicated, but at least the expression in neural tube can be described in relation to the adjacent somites. This reveals that *AmphiHox-3* has an anterior expression limit at around the level of somite 4; in other words, amphioxus has four somite anterior pairs rostral to the *AmphiHox-3* domain. Recent work suggests this is more accurately described as 'three and a half' somite pairs rostral to the *AmphiHox-3* domain (Wada *et al.*, 1999). There is some debate as to whether there is a fifth segment in the most anterior tip of amphioxus embryos, since the most rostral part of the archenteron (primitive gut) has diverticula that some argue reflect a modified terminal segment (Presley *et al.*, 1996). I am unconvinced, and maintain that there are three and a half mesodermal segment pairs anterior to the *AmphiHox-3* domain.

How does this expression compare with vertebrates? First, we must satisfy ourselves that such comparison is valid. After all, vertebrates do not possess just a single gene that is directly orthologous to *AmphiHox-3*; rather, they have three equally related genes descendent from *Hox* gene cluster duplication. Fortunately, these three genes *(Hoxa-3, Hoxb-3 and Hoxd-3)* all have identical anterior expression positions in the developing mouse neural tube (and in other vertebrates where examined), suggesting they inherited this aspect of their regulation from their precursor gene (an extinct ortholog of *AmphiHox-3)*. Further, there is no clear evidence that group 3 *Hox* genes of amphioxus or mouse have undergone substantial change in their regulation in relation to other *Hox* genes. The same might not be said so readily for group *Hox 1* genes, for example, which do not respect the spatial colinearity rule in the mouse; recent data suggest this deviation from colinearity is as a secondary phenomenon (Wada *et al.*, 1998, 1999). I suggest, therefore, that it is valid to compare the anterior limit of *AmphiHox-3* expression in amphioxus with the anterior limit of *Hoxa-3*, *Hoxb-*

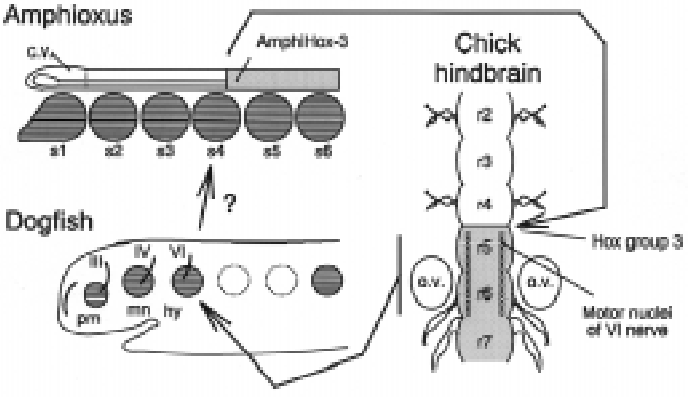

Fig. 2. Putative homologies between rostral regions of amphioxus, chick and dogfish. *The amphioxus diagram shows nerve cord and somites; chick shows hindbrain only; dogfish shows putative mesodermal head segments and some cranial nerves (simplified from Goodrich, 1930). The dotted circles in the dogfish diagram are segments inferred to have been lost in vertebrate evolution, according to Goodrich. The rostral limit to Hox group 3 expression is compared between amphioxus and chick neural tubes (top arrow); since the equivalent gene(s) has not yet been described in dogfish, the position is compared to dogfish using the VI nerve as a landmark (bottom arrow). The VI nerve is associated with Goodrich's third mesodermal segment of the dogfish head; this may therefore be comparable to the fourth mesoderm segment of amphioxus (arrow with question mark). The evolutionary implications of this comparison are discussed in the text. Abbreviations: III, IV, VI, cranial somatic motor nerves; c.v., cerebral vesicle; o.v., otic vesicle; pm, premandibular head cavity; mn, mandibular head cavity; hy, hyoid head cavity; r2-7, rhombomeres; s1-6, somites.*

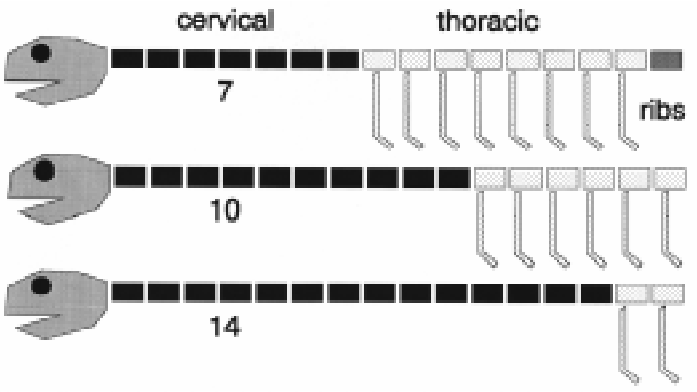

Fig. 3. Simplified skeletons of three hypothetical vertebrates indicating the principle of transposition.

3 and *Hoxd-3* expression in mouse embryos. The genes could be used as 'molecular landmarks' revealing the homologous position.

In mouse and chick embryos, the Hox paralogy group 3 genes have an anterior expression limit within the hindbrain, at the boundary between the fourth and fifth rhombomeres. Is this consistent with the Goodrich model of vertebrate head segmentation, with this in turn being a simple modification of the amphioxus condition? The problem is that for vertebrates, we cannot simply count how many of Goodrich's hypothesized mesodermal segments lie rostral to this gene expression point, since segmentation is far from clear in the cranial paraxial mesoderm, and since his model suggested that some segments have been lost in evolution. We can make the comparison to Goodrich's model using cranial nerves, however. It is sensible to restrict our attention to the somatic motor nerves, since these innervate the striated muscles purported to derive from the cranial mesodermal segments. These include the oculomotor (III) nerve, the trochlear (IV) nerve and the abducens (VI) nerve; in Goodrich's model, these innervate mesodermal segments 1, 2 and 3. Lumsden and Keynes (1989) demonstrated that in the chick hindbrain, the third of these (abducens) exits from rhombomere 5, just behind the Hox 3 expression limit. Thus, the rostral expression limit for Hox paralogy group 3 genes of chick or mouse lies opposite putative segment three of Goodrich's vertebrate head groundplan (Fig. 2).

Hence, gene expression data suggest that a position opposite mesodermal segment four of amphioxus is homologous to a position opposite mesodermal segment three of Goodrich's early vertebrate. This is a reasonably close correlation, but some explanation must be sought for the slight numbering difference. One possibility is that vertebrates have an extra segment rudiment rostral to the premandibular, not recognized by Goodrich. The existence of such a segment has been proposed previously on strictly morphological grounds; conceivably an additional putative head cavity, Platt's vesicle, may be a remnant of this segment (but see Horder *et al.*, 1993, for an opposing view). Alternatively, perhaps the relationship between cranial nerves and somites are not constrained to respect a single segmental series, meaning that our extrapolation from neural expression to somites is flawed. Finally, the Goodrich model could be a perfectly accurate reflection of the vertebrate head groundplan, but the numbers of cranial segments have changed on either the amphioxus or vertebrate lineages since their divergence. Each explanation is feasible, although other gene expression patterns are needed to resolve the matter. One gene analyzed recently is the homeobox gene *Otx*, thought to mark the forebrain and midbrain. Comparison of the amphioxus *Otx* gene, *AmphiOtx*, to one of its two mouse homologs, *Otx2,* has helped to identify a putative forebrain region in the amphioxus neural tube; its position is perfectly compatible with the Hox data (Williams and Holland, 1996). To complete the assessment of Goodrich's model, what is needed now is analysis of amphioxus and vertebrate genes spatially expressed in cranial mesoderm and the gill support tissues.

In summary, recent molecular data are generally supportive of Goodrich's model of head segmentation, although rostral segment numbers may have changed slightly in either the lineage leading to amphioxus or that to dogfish, or he may have overlooked a segment. With regard to the former suggestion, it is worth noting that recent scanning electron microscopy studies suggest that mesodermal segment numbers have also changed between groups of vertebrates. Specifically, teleosts may have secondarily subdivided some of the rostral mesodermal segments (Gilland and Baker, 1993; Jacobson, 1993).

Transposition and skeletal pattern

In contrast to the conserved segmental organisation proposed for the head of most vertebrates, differentiation along the postcranial skeleton shows considerable variation between close species; a point again stressed by Goodrich (1906, 1913). For example, the pectoral (or pelvic) girdles can be located at a variety of axial positions in tetrapods, as judged by counting vertebral number. Similarly, major transitions in vertebral morphology, such as that from cervical (neck) vertebrae to thoracic (rib-bearing) vertebrae, can be located at very different axial positions (Fig. 3). This migration of phenotype up or down a segmental series during evolution was termed 'transposition' by Goodrich (1906, 1913). In his words "structure and segmentation vary independently, and whatever may be the connection which becomes established between them, and however close it may be, it would seem that we must not consider it constant" (Goodrich, 1913).

As a consequence of research in developmental biology, Goodrich's elusive "connection" between structure and segmentation has now come within grasp. As outlined in the previous section, Hox genes are implicated in interpreting position along the body axis (which segment am I in?) and translating this into structure (what should I become?). Evidence was first obtained from *Drosophila*, where *Hox* genes play the pivotal role in specifying correct segmental identity in insect embryos (Lewis, 1978; Gehring, 1987). In vertebrates, indirect support that *Hox* gene expression controls vertebral identity comes from the observation that the sharp limits to expression of particular *Hox* genes often correlate with morphological changes in vertebral identity along the axial skeleton (Kessel, 1991). Direct support has come from mice in which the axial Hox code has been experimentally altered during development, either by retinoic acid treatment in pregnancy (Kessel and Gruss, 1991), ectopic expression of *Hox* transgenes (Kessel *et al.*, 1990; Lufkin *et al.*, 1992) or inactivation of endogenous *Hox* genes (e.g., Le Mouellic *et al.*, 1992; Jeannotte *et al.*, 1993; Fromantal-Ramain *et al.*, 1996; Saegusa *et al.*, 1996). Although the phenotypes produced by such modifications are complex, it is clear that

alteration of the Hox code can cause alteration of vertebral identities (for example, a shift in the boundary between vertebral types).

These experiments confirm that shifts in *Hox* gene expression patterns are capable of causing shifts in vertebral formulae, in an experimental setting. They do not prove that shifts in *Hox* gene expression patterns have actually caused shifts in vertebral formulae during evolution. Recent studies, however, by Stephen Gaunt in Cambridge, England, and by Annie Burke, then at Harvard University, USA, provide strong evidence that this is indeed the case (Gaunt, 1994; Burke *et al.*, 1995). In both studies, *Hox* gene expression limits were compared between species with distinct vertebral formulae (e.g., chick versus mouse). The data clearly indicate that the Hox code correlates with vertebral identity (e.g., cervical versus thoracic), not with vertebral position (somite number). This link is probably causal, in view of the functional data outlined above. Hence, the phenomenon termed transposition by Goodrich is now explainable at the genetic level: transposition in vertebral formulae results from spatial shifts in *Hox* gene expression patterns during vertebrate evolution.

Conclusions and prospects

The first two subjects discussed above, the nature of amphioxus and segmentation of the vertebrate head, have long been contentious issues in biology. Goodrich was just one of many scientists to have put forward a view on each of these topics. Recent findings in developmental biology have allowed us to revisit these topics with new and more definitive evidence; in each case, Goodrich's views are being confirmed as largely accurate. The third topic explored, transposition, was not a subject of heated debate; it was a novel and insightful observation made by Goodrich alone. In this case, modern findings have not tested a hypothesis of Goodrich, instead they have built upon the foundation that he laid down, adding a mechanistic slant.

The recent data discussed have certainly increased our understanding of chordate evolution, head development, segmentation, serial homology and vertebral column development, but they have also left some questions unanswered and opened some potential new lines of inquiry. A few of these are worth highlighting. With respect to the nature of amphioxus, the key problems remaining are to resolve just how closely amphioxus resembles a direct predecessor of vertebrates, and to understand the evolutionary processes that transformed such an ancestor into a vertebrate. Turning to head segmentation, it is only the first step to deduce the number and position of ancestral segments in the head. The next step must be to elucidate the stepwise transformations that converted the ancestral series of mesodermal segments into the highly modified head. Furthermore, there is still the open question of whether the branchial arches were originally part of the same metameric series as paraxial mesoderm segments, or whether they are an independent series superimposed onto the head during evolution.

The demonstration that *Hox* genes probably lie at the heart of transposition is a major breakthrough, and neatly closes the link between position and structure highlighted by Goodrich. But there is another aspect to transposition that Goodrich dismissed, but which I suspect will come to be its most fascinating aspect. This is the presence of phylogenetic patterns in the proclivity for transposition. Goodrich (1913) noted that not every part of the axial skeleton is subject to transposition in every vertebrate taxon. Examples cited by Goodrich include the constancy of anterior head segmentation in craniates, the fact that the last lumbar vertebra of artiodactyls (deer, camels, pigs etc.) is always the twenty sixth, and the near constancy of seven cervical vertebrae across mammals. Although Goodrich (1913) acknowledged that these exceptions were "remarkable", he nonetheless dismissed them as "of secondary importance". I disagree. Such exceptions to evolutionary variation raise important (e.g. see Galis, 1999) questions. Are there developmental or genetic constraints to transposition in certain lineages? Or do the exceptions reflect the optimal adaptive condition for particular taxa? Are there constraints to transposition in certain body regions? If spatial shifts in *Hox* gene expression cause transposition, why do these occur in some lineages and not others? Are there different mechanisms of *Hox* gene regulation in different vertebrate taxa, such that bird and reptilian *Hox* gene regulation is rather easily altered but mammalian Hox regulation more resistant to change? Several of these questions are amenable to experimental testing; their investigation will be extremely informative to both developmental and evolutionary biology.

KEY WORDS: *vertebrate head, amphioxus, Hox, evolution*

Acknowledgments

I thank Michael Hardy for the gift of a wonderful collection of Goodrich's reprints, the Department of Zoology, University of Oxford, for allowing me to reproduce the photograph of Goodrich, members of my lab for their work on amphioxus, Anthony Graham and Per Ahlberg for discussions, and Jim Smith for the invitation to contribute an article for this issue.

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