Building the backbone: the development and evolution of vertebral patterning

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ABSTRACT

The segmented vertebral column comprises a repeat series of vertebrae, each consisting of two key components: the vertebral body (or centrum) and the vertebral arches. Despite being a defining feature of the vertebrates, much remains to be understood about vertebral development and evolution. Particular controversy surrounds whether vertebral component structures are homologous across vertebrates, how somite and vertebral patterning are connected, and the developmental origin of vertebral bone-mineralizing cells. Here, we assemble evidence from ichthyologists, palaeontologists and developmental biologists to consider these issues. Vertebral arch elements were present in early stem vertebrates, whereas centra arose later. We argue that centra are homologous among jawed vertebrates, and review evidence in teleosts that the notochord plays an instructive role in segmental patterning, alongside the somites, and contributes to mineralization. By clarifying the evolutionary relationship between centra and arches, and their varying modes of skeletal mineralization, we can better appreciate the detailed mechanisms that regulate and diversify vertebral patterning.

KEY WORDS: Bone, Notochord, Sclerotome, Segmentation, Vertebrae

Introduction

The vertebral column is the defining feature of vertebrates and comprises a segmented/repeat series of individual bones, the vertebrae. These possess two fundamental features: the vertebral body, or centrum, which envelops the notochord to provide axial mechanical strength, and the dorsal and ventral vertebral arches, which enclose and protect, respectively, the spinal cord and axial blood vessels (Fig. 1). It should be noted, however, that a variety of anatomical terms have been used to describe vertebral sub-components and their mineralization, with some accompanying historical confusion (reviewed in Box 1).

The crucial role played by somite segmentation in vertebral development and patterning has long been recognized, starting with Robert Remak’s pioneering observations in the chick embryo (Remak, 1855). Many recent studies have elucidated the molecular mechanisms that control how somite boundaries form, and that regulate the oscillatory gene expression underlying somite formation (comprehensively reviewed by Bénazéraf and Pourquié (2013); Delaune et al. (2012); Lewis et al. (2009); Nakashima et al., 2002; Peters et al., 1999). It is not clear, however, whether this description holds for the centra of all vertebrates, particularly in anamniotes (fishes and amphibians), in which the sclerotome comprises only a small subset of cells in each segment. Furthermore, in teleosts, the centra and most arches form by direct ossification without a cartilage template (Box 2). Here, we review evidence suggesting that vertebral mineralizing cells in some fish have non-somite origins. These studies further imply that the somites might not be solely responsible for generating vertebral segmental patterning, as commonly assumed, and reveal significant phylogenetic diversity in the mechanisms that mineralize the vertebrae. We also consider the relationship between somite segmentation and vertebral segmentation, and its variation within different vertebrate lineages.

Vertebral arches might have evolved first in the ancestral cranialite

Craniates are chordates with a skull (Janvier, 1996) and comprise agnathans (jawless fish) and gnathostomes (jawed vertebrates; Fig. 2). There are two extant groups of agnathans: the lampreys and the hagfish, collectively termed cyclostomes. Lampreys are well known to possess cartilaginous vertebral arch elements, positioned dorsally and periodically along the notochord, in register with the myotomes [see e.g. Goodrich (1930)] and corresponding to the vertebral arches of gnathostomes. These presumably originate from sclerotome cells, as indicated by recent studies showing the expression patterns of genes proposed as sclerotome markers (see Table 1), such as FoxC2, Tbx18 and the orthologue of the ancestor of scleraxis (Freitas et al., 2006), and Col2a and Sox9 (Zhang, 2009). Until recently, the other extant
Markers associated with cells that express orthologues of the sclerotome features with the vertebral elements of other vertebrates and are hagfish species (Ayers and Jackson, 1901). Recent reinvestigation nodules positioned ventral to the notochord in the tail of some from the end of the 19th century identified minute cartilaginous hence the formal classification of Vertebrata as a subdivision of agnathan group, the hagfish, were thought to have no vertebrae; because of its remarkable idealism [see especially Gadow (1933)], and was challenged repeatedly during its long history. It was eventually dismissed, or found not useful, for one vertebrate group after another – tetrapods (Williams, 1959), the acahnthians (Miles, 1970) and osteichthyans (Schaeffer, 1967), and particularly the dinoans (lungfish) (Arratia et al., 2001). More recent work reviewed here provides no evidence for Gadowian organization of the centrum within teleosts. Nonetheless, the hypothesis has not completely disappeared, particularly from the palaeontology literature, and has caused a persistent confusion in terminology. Its widespread acceptance throughout much of the 20th century has probably resulted in the shoehorning of experimental findings to fit a hypothesis that now seems to have lost any usefulness.

Box 1. Gadow’s arcualia

Early work by Gadow (1896) and by Gadow and Abbott (1895) led Gadow to a hypothesis that markedly influenced understanding of vertebral development and terminology. The ‘Gadowian’ hypothesis proposed that, across all vertebrates, four bilaterally paired primordia called ‘arcualia’ give rise to different parts of the vertebra. The dorsal ‘basidorsals’ and ventral ‘basiventrals’ form the vertebral arches, respectively the neural arches (enveloping spinal cord) and haemal arches (enveloping axial blood vessels), as well as the regions of the centra growing out (usually perichordally) from the arch bases. Alternating with these two along the body axis, and completing the segmental composition of a single centrum, are interdorsals and interventrals. However, this view of a vertebra, appealing in the way it proposes a basic uniformity among diverse vertebrates, is worrisome – as a hypothesis that markedly influenced understanding of vertebral development and terminology. The ‘Gadowian’ hypothesis proposed that, across all vertebrates, four bilaterally paired primordia called ‘arcualia’ give rise to different parts of the vertebra. The dorsal ‘basidorsals’ and ventral ‘basiventrals’ form the vertebral arches, respectively the neural arches (enveloping spinal cord) and haemal arches (enveloping axial blood vessels), as well as the regions of the centra growing out (usually perichordally) from the arch bases. Alternating with these two along the body axis, and completing the segmental composition of a single centrum, are interdorsals and interventrals. However, this view of a vertebra, appealing in the way it proposes a basic uniformity among diverse vertebrates, is worrisome – because of its remarkable idealism [see especially Gadow (1933)], and was challenged repeatedly during its long history. It was eventually dismissed, or found not useful, for one vertebrate group after another – tetrapods (Williams, 1959), the acahnthians (Miles, 1970) and osteichthyans (Schaeffer, 1967), and particularly the dinoans (lungfish) (Arratia et al., 2001). More recent work reviewed here provides no evidence for Gadowian organization of the centrum within teleosts. Nonetheless, the hypothesis has not completely disappeared, particularly from the palaeontology literature, and has caused a persistent confusion in terminology. Its widespread acceptance throughout much of the 20th century has probably resulted in the shoehorning of experimental findings to fit a hypothesis that now seems to have lost any usefulness.

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Centra arose within gnathostomes

Mineralized centra are present in almost all of the major lineages of Euphanerops, a group comprising all jawed vertebrates except placoderms (Fig. 2). The three major living gnathostome groups – chondrichthysans, actinopterygians and sarcopterygians (including tetrapods) – all have examples of species that possess centra. Such a distribution of centra among these ‘crown group’ gnathostomes immediately suggests that centra should be considered as primitive
Sclerotome markers

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<th>Gene</th>
<th>Caveats</th>
<th>References</th>
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<tr>
<td><strong>Twist</strong></td>
<td>Chick/mouse – not restricted to the sclerotome lineage and expressed in other tissues in the trunk (neural crest, trunk mesoderm)</td>
<td>(Barnes and Fiuill, 2009)</td>
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<td></td>
<td>Zebrafish – 2 orthologues, <strong>Twist1a</strong> and <strong>Twist1b</strong>, expressed in separate domains</td>
<td>(Yeo et al., 2009)</td>
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<td></td>
<td>Medaka – sclerotome and ‘putative’ neural crest</td>
<td>(Yasutake et al., 2004)</td>
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<td><strong>Pax9</strong></td>
<td>Mouse – expression restricted to the dorsal half of sclerotome and in non-somatic tissues</td>
<td>(Peters et al., 1999)</td>
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<td></td>
<td>Teleosts – expression throughout the sclerotome</td>
<td>(Peters et al., 1998)</td>
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<td><strong>Pax1</strong></td>
<td>Chick – early marker of ventral sclerotome</td>
<td>(Christ et al., 2004)</td>
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<td></td>
<td>Medaka – general sclerotome marker</td>
<td>(Mise et al., 2008)</td>
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<tr>
<td></td>
<td>Zebrafish – 2 orthologues, <strong>Pax1a</strong> and <strong>Pax1b</strong></td>
<td>(*<a href="http://zfjin.org">http://zfjin.org</a>)</td>
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<tr>
<td><strong>Foxc2</strong> (Mfh1, cFkh1)</td>
<td>Chick – expressed in the paraxial mesoderm prior to somite formation, then expression restricted within the sclerotome to a domain more dorsal and lateral to that of Pax1</td>
<td>(Christ et al., 2004)</td>
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<td></td>
<td>No homologue identified in teleosts</td>
<td>(*based on homology information in Homologene database: <a href="http://www.ncbi.nlm.nih.gov/homologene">http://www.ncbi.nlm.nih.gov/homologene</a>)</td>
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<td><strong>Scleraxis</strong></td>
<td>Mouse and chick – expressed in part of sclerotome that gives rise to tendons and ligaments</td>
<td>(Perez et al., 2003)</td>
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<td></td>
<td>Zebrafish possess 2 orthologues</td>
<td>(Christ et al., 2004)</td>
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<tr>
<td><strong>Zic1</strong></td>
<td>Chick – sclerotome expression but also nascent myotome and non-migratory neural crest</td>
<td>(Sun Rhodes and Merzdorf, 2006)</td>
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<td></td>
<td>Mouse – dorsal sclerotome and dermomyotome</td>
<td>(Aruga et al., 1999)</td>
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<td></td>
<td>Xenopus – neural crest development</td>
<td>(Rohr et al., 1999)</td>
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<td></td>
<td>Zebrafish – dorsal somite, partially overlapping with myotome</td>
<td>(Moriyama et al., 2012)</td>
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<td></td>
<td>Medaka – dorsal somite</td>
<td></td>
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<tr>
<td><strong>Tbx18</strong></td>
<td>Mouse – initially anterior half-somite, then just sclerotome and non-somatic domains</td>
<td>(Kraus et al., 2001)</td>
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<tr>
<td></td>
<td>Zebrafish – initially anterior half somite, then around horizontal myoseptum</td>
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Differentiation markers of sclerotome/chondrocytes/osteoblasts

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<tr>
<th>Gene</th>
<th>Caveats</th>
<th>References</th>
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<tr>
<td><strong>Sox9</strong></td>
<td>Chondrocyte marker, including those derived from cranial neural crest</td>
<td>(Mori-Akiyama et al., 2003)</td>
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<td></td>
<td>Expressed in the notochord in mouse and zebrafish</td>
<td>(Barnounevo et al., 2006)</td>
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<td></td>
<td>2 orthologues in zebrafish</td>
<td>(Yan et al., 2005)</td>
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<tr>
<td><strong>Col2a</strong></td>
<td>Similar expression to Sox9</td>
<td>(Zhao et al., 1997)</td>
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<tr>
<td><strong>Col10a1</strong></td>
<td>Mouse – chondrocytes</td>
<td>(Avaron et al., 2006)</td>
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<td></td>
<td>Zebrafish – in osteoblasts of bones formed by direct ossification</td>
<td>(Renn et al., 2013)</td>
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<tr>
<td></td>
<td>Medaka – in some of sclerotome-derived but also expressed in other vertebrae-forming cells of unknown origin</td>
<td></td>
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<tr>
<td><strong>Sp7 (Osterix)</strong></td>
<td>Early osteoblast marker</td>
<td>(Nakashima et al., 2002)</td>
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<td>(Li et al., 2009)</td>
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In some developmental studies, expression of a single gene is used to define a cell population (e.g. sclerotome or osteoblasts). However, in many cases, these markers are not unique to the population they are being used to identify. Here, we have provided evidence for whether the genetic markers cited in the text are faithful markers of the cell population they are being used to study and the caveats for interpreting these findings. This is not an exhaustive list of all sclerotome or osteoblast markers but is used to highlight the problems with interpreting data when tracking a migratory cell population in which expression profiles change over time.

within the Eugnathostoma. However, whereas the presence of mineralized centra is widespread within the group, it is also patchy (Fig. 2), such that within each of the major sub-lineages there are branches that do not form centra of any type. For example, sturgeons (actinopterygians) and paddlefish (chondrosteans) have complex mineralized vertebral arches but no centra. Rather, their notochord possesses a thick fibrous sheath that supports the body and onto which the dorsal and ventral ossified vertebral arches articulate. A similar morphology is present in the sarcopterygians, from which tetrapods descended; the ‘living fossil’ coelacanth Latimeria has arches but no centra (Arratia et al., 2001). The only group of eugnathostomes that shows no evidence of centra comprises the acanthodians, an extinct lineage known only from fossils. It is possible, however, that some acanthodians possessed non-mineralized centra that would not have been preserved during fossilization, and hence would not be recognizable. Indeed, a transition from a mineralized to non-mineralized state is seen in the evolution of the skeleton of chondrichthyans (Orvig, 1951).

The inconsistent presence of centra among the gnathostomes led Arratia and colleagues, in a detailed and influential paper (Arratia et al., 2001), to reject the concept of homologous centra, proposing instead that centra were not present in the stem eugnathostome and that they arose independently in multiple subsequent lineages. However, centra might have been more easily lost during evolution than re-invented multiple times, and we suggest that multiple loss events is a more parsimonious interpretation of the available phylogenetic evidence. Where a trait is widespread but not ubiquitous within a group, West-Eberhard (2003) has proposed the concept of ‘broad-sense homology’ (see Box 3) to account for recurrence or independent parallel evolution from an ‘ancestral developmental propensity’ (West-Eberhard, 2003). Within this framework, centra can be considered homologous with one another.
Box 2. Methods of bone formation

**Acellular bone**: Bone formed by the polarised secretion of osteoid from osteoblasts that remain at the bone surface and do not become embedded (i.e. do not become osteocytes). This is the typical bone found in tetrapods.

**Calcified cartilage**: Tissue that forms where the extracellular matrix of cartilage becomes mineralized with hydroxyapatite. Cartilage cells persist within this matrix and no osteoblasts are present.

**Endochondral ossification**: The process of bone formation from an existing cartilage template; the shape of the bone is initially formed from cartilage and is then replaced by bone. This process is typical in the axial skeleton and limb bones of mammals. Bones formed in this way are termed chordoid or chondral bones.

**Intramembranous ossification**: The process by which bone forms in the absence of a cartilage template, i.e. by direct ossification. During this process, bone forms without being in contact with ectoderm or endoderm. This process is typical in the skull bones of mammals and in the scales and fin rays of fish. Bones formed in this way are referred to as intramembranous, dermal or membrane bone, depending both on the species and the anatomical location. [Note: Patterson (1977) used the terms dermal and membrane bone to describe different modes of direct ossification.]

**Ossification**: The process of bone formation whereby osteoblasts (bone cells; see below) secrete and become surrounded by a matrix that becomes mineralized with hydroxyapatite and calcium carbonate.

**Osteoblasts**: Bone-forming cells. In some forms of bone formation, these cells become embedded in the matrix they secrete and are then termed osteocytes. Some authors also refer to atypical osteoblasts, namely bone-secreting cells that do not express certain genes that have previously been described for this lineage (see Table 1).

**Osteoid**: Recently deposited unmineralized bone that can be cellular (containing osteocytes) or acellular (no cells embedded within it).

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**Centra subtypes and their formation: insights into the evolution of vertebrates**

There are several subtypes of centra, defined according to morphology [reviewed by Arratia et al. (2001)], and here we focus on the distinction between ‘chordacentra’ and ‘perichordal’ centra. In most tetrapods, and in some fish, centra develop only perichordally – around and external to the notochord and its sheath. However, in many species within two major groups of fish, elasmobranchs (which include sharks, rays and skates) and teleosts (ray-finned fish, including zebrafish and medaka), the early-developing centra, known as chordacentra, first form as ring-shaped mineralizations within the fibrous collagen-rich sheath of the notochord (Gadow and Abbott, 1895; Grotmol et al., 2006). In these fish, a perichordal centrum forms secondarily.

**Ossification of the perichordal centrum**

In considering whether centra are homologous, it is necessary to consider the processes of ossification within different lineages and what they may reveal about the evolution of this structure. In elasmobranchs and tetrapods, the perichordal centrum forms by endochondral ossification (Box 2), whereas in teleosts it is formed by direct ossification (Box 2) without a cartilage intermediate (Bensimon-Brito et al., 2012). This difference does not necessarily argue for non-homology between the perichordal centra of teleosts and other vertebrates. But, as clearly proposed by De Beer (1930), and then in much more detail by Patterson (1977), the difference does represent a radical change in how putative homologous elements (in this case centra) can develop. Patterson hypothesized that, in a primitive common ancestor of both groups, bones developed in one of only two ways, either as dermal bones in the skin mesenchyme (dermal bones of the exoskeleton) or in close association with cartilage (chondral bones of the endoskeleton). In derived lineages, cartilage development might regress but their once-associated bones persist. Such ‘membrane bones’ of this third type, as Patterson termed them, formed by direct ossification, are frequently encountered in derived forms and in locations other than the vertebral column (e.g. the teleost skull; Patterson, 1977). Although the naming scheme for distinguishing the two types of direct ossification has not caught on, his proposal fits perfectly with broad-sense homology, and is well worth pursuing in studies of the mineralized tissue, especially in teleosts.

**Formation of the chordacentrum**

Of further interest in a comparative context are the processes by which chordacentra form in elasmobranchs and teleosts. The elasmobranch chordacentra are usually described as composed of ‘calcified cartilage’ [e.g. Dean and Summers (2006); Peignoux-Deville et al. (1982)], consisting of hydroxyapatite deposited in the extracellular matrix of the cartilage. The cells and matrix of the original cartilage persist in the calcified tissue (Ridewood, 1921) and produce mineralized matrix (Hall, 2005). This mineralized

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**Fig. 2. The phylogeny and appearance of vertebral elements in vertebrate phyla.** Vertebral arches are thought to have arisen in the stem group that gave rise to agnathans and gnathostomes, whereas centra appear later and are only present in gnathostomes. Mineralized vertebral elements have been identified in the fossil *Euphanerops* found in the late Devonian that lived about 380 million years ago. Fossils of *Haikouichthys* are found in the Cambrian, ~525 million years old. Vertebrate groups are sometimes subdivided into amniotes (reptiles, birds and mammals) and amniotes (fishes and amphibians).
tissue is histologically different from that found in the elasmobranch vertebral arches (Eames et al., 2007; Orvig, 1951), which consist of bone that forms around and eventually replaces the cartilage template (Peignoux-Deville et al., 1982). Whereas elasmobranch arches and centra therefore clearly differ in their mode of formation, the terms used to describe the process and properties of the mineralization of different skeletal components in all fishes might have been oversimplified [see Eames et al. (2007) for a more detailed review of this area], perhaps reflecting the pigeonholing of fish mineralized skeleton into terms already described for mammals and birds. However, the clear distinction between elasmobranch chordacentrum calcified cartilage and arch bone is important, as it allows for comparison with teleosts. Chordacentra in teleosts form by the deposition and subsequent mineralization of matrix within the collagen-rich notochord sheath. This process is similar to that occurring in elasmobranchs, in which the cartilage itself is calcified rather than being replaced by bone. Indeed, there is evidence that the teleost chordacentrum mineral is hydroxyapatite (Wang et al., 2013), matching both bone and calcified cartilage in this respect (Hall, 2005).

Cellular origins of chordacentra

In elasmobranchs, the chordacentrum mineralizes as a layer within cartilage that, in turn, is contained within a notochordal sheath that earlier in development was acellular, consisting only of a fibrous connective tissue-like extracellular matrix. The source of the cartilage cells, assessed in many descriptive studies using sectioned material, is likely to be the sclerotome but the evidence is incomplete (Arratia et al., 2001; Goodrich, 1930). If correct, sclerotome cells would have to navigate through the outermost fibrous component of the notochord sheath, the elastica externa, which is widely present in gnathostome fishes. Indeed, histological evidence from Scyllium canicula (Goodrich, 1930), suggested by Goodrich to be representative of all elasmobranchs (Fig. 3), shows that this layer does become perforated and that cells are sometimes present within the perforations, perhaps caught in the act of migration. Likewise, in dipnoans (e.g. lungfish) and sturgeons (Arratia et al., 2001; Goodrich, 1930), cartilage cells are present within the fibrous notochordal sheath and are hypothesized to have arrived there by migration through the perforated elastica externa. In sturgeons and most of the vertebral column of dipnoans, however, centra do not form. The putative existence of these similar migration patterns in diverse fish lineages, regardless of whether they form centra, presents an intriguing problem with respect to centrum homologies among gnathostomes. By contrast, there is no evidence for invasion of the teleost notochord sheath by sclerotome cells in the three species in which vertebral development has been experimentally investigated in detail, namely medaka (Oryzias latipes), Atlantic salmon (Salmo salar) and zebrafish (Danio rerio).

Given the apparent absence of sheath invasion in teleosts, the question arises as to how their chordacentra mineralize. Electron micrographs of medaka and Atlantic salmon show that chordacentra form within the notochordal sheath before the perichondral centrum (Ekanayake and Hall, 1988; Nordvik et al., 2005). Whereas the cells that secrete the perichondral bone matrix are on the outer vertebral surface and have been verified as sclerotome cells (Inohaya et al., 2007), the cells responsible for the initial chordacentrum osteoid secretion in the electron micrographs were not identified. Indeed, several observations suggest that the cells giving rise to this initial sheath-associated osteoid matrix are not typical bone matrix-secreting cells (osteoblasts, see Box 2) and are not derived from the sclerotome. First, there is no evidence that mature osteoblasts are present at the time of chordacentrum formation. In zebrafish,

Box 3. Broad-sense homology

Homology in a strict cladistics sense requires that, if traits in different taxa are to be considered homologous, their expression in the monophyletic group including these taxa must be unbroken, i.e. the trait must be present in all of the members of the monophyletic group. Such cladistic homology might be essential for systematics, but is over-restrictive if the subject of interest is evolutionary change within the gnathostomes, rather than the relationships between gnathostomes (West-Eberhard, 2003).

There are various terms for different types of homology, such as deep homology (Shubin et al., 2009) or Wagner’s special and general homology (Wagner, 2014), each of which have nuanced differences in definition and are useful for describing different concepts. Homology in the ‘broad sense’ as described by West-Eberhard (2003) and similar to what Roth (1991) means by biological, or transformational, homology is particularly useful for understanding the evolution of development because it admits such features as latent homologies and recursive appearances of homologous characters in a lineage. Broad-sense homology ‘allows’ homologous traits to be lost among members of a clade and then to reappear sporadically (recur) among their descendants. Recurrence in such a restricted phylogeny is not expected to be solely by a brand-new and independent invention of the trait, but to depend on close ancestry. Hence, the concept of broad-sense homology has some real meaning in evolutionary biology. On the basis of broad-sense homology, we suggest that a hypothesis of centrum homology among eugnathostomes should not be dismissed.
osteoblasts identified by the expression of \textit{sp7} (see Table 1), and presumably of sclerotomal origin, are only detected at the onset of vertebral arch formation, whereas chordacentra begin mineralizing much earlier in development [5 versus 17 days after fertilization; Spoorendonk et al. (2008)]. When \textit{sp7}+ cells are observed, they are located only at the arches and peripheral to already-established chordacentra. Second, functional evidence indicates that sclerotome-derived cells are not necessary for the initial formation of segmental chordacentra. When \textit{sp7}+ cells are genetically ablated in medaka, segmented chordacentra first develop normally, but then fuse at later stages to form an unsegmented rod of bone. Additionally, these fish do not develop vertebral arches, an observation also consistent with an exclusive role for the sclerotome in arch and perichordal centrum formation (Willems et al., 2012).

How, then, does the teleost chordacentrum form? A recent study using reporter transgenic medaka lines has identified a possible \textit{sp7}+ cell population expressing \textit{col10a1} at the nascent chordacentra, and there is some evidence that part of this population is sclerotome derived (Renn et al., 2013). However, morpholino studies targeting sclerotome cells earlier in their differentiation suggest that segmented centra can form independently of sclerotome cells. For example, knockdown of \textit{twist} in medaka results in aberrant vertebral arches but normal centrum development (Yasutake et al., 2004). Likewise in single and double knockdowns of \textit{pax1} and \textit{pax9}, centrum formation is initially normal but vertebral arches fail to form and scoliosis develops later (Mise et al., 2008). Such partial disruption of vertebral column development could arise from compensatory mechanisms or incomplete gene knockdown, and at present there are no published null mutant analyses of these genes.

Overall, current evidence indicates that the cells generating teleost chordacentra are neither typical osteoblasts nor necessarily sclerotomal in origin. Additionally, the teleost notochord has been identified as a source of chordacentrum mineralization. In support of this, it was shown that zebrafish notochords grown in \textit{ex vivo} culture secrete an osteoid matrix, and laser ablation of single notochord cells \textit{in vivo} at segmentally repeated axial positions results in loss of chordacentra at these positions (Fleming et al., 2004). These studies do not, however, define the precise notochord cell population responsible for mineralization. A good candidate is the outer epithelial layer of ‘chordoblast’ cells that secretes the fibrous notochord sheath, as alkaline phosphatase activity, which is thought to be essential for mineralization (Hessle et al., 2002), has been detected in these cells in developing Atlantic salmon (Grotmol et al., 2005).

**An instructive role for the notochord**

Such a role for the notochord is supported by studies of the zebrafish fused somites/tbx6 mutant. Here, somites show abnormal segmentation and disrupted sclerotome patterning, resulting in disorganized vertebral (neural and haemal) arch formation, yet mutants retain normal centrum segmentation (Fleming et al., 2004; Nikaido et al., 2002; van Eeden et al., 1996). Consistent with this, Atlantic salmon notochord sheath cells (chordoblasts) re-orient metamerically in register with chordacentrum formation (Grotmol et al., 2003), correlating with their segmental expression of alkaline phosphatase activity (Grotmol et al., 2005). Moreover, whereas there is currently no strong evidence indicating that the notochord was ancestrally segmented (Stern, 1990), a segmented notochord architecture has been tentatively identified in the fossil protvertebrate \textit{Pikaia} (Conway Morris and Caron, 2012). The recent suggestion that the notochord evolved from contractile axial mesoderm cells with segmental connections to transverse muscles in a bilaterian ancestor (Lauri et al., 2014) adds further credence to this view. It might also be significant that segmental cartilage formation within the notochord has been detected in light microscope studies of centrum development in urodele and apodan amphibia, \textit{Sphenodon} and many Lacertilia (Goodrich, 1930; Lawson, 1966; Mookerjee, 1930), raising the possibility that a similar notochord/sclerotome duality in centrum development existed in stem tetrapods.

**Resegmentation in amniotes**

In amniotes, the registration between centra and somites changes during development (Fig. 4), and since Remak’s initial observations (Remak, 1855) this has been widely accepted to be due to a frameshift or ‘resegmentation’ [see Verbout (1976) for a review of the early studies]. Remak saw that the chick sclerotome is polarized into anterior (A, cranial) and posterior (P, caudal) halves, the anterior half containing the spinal nerves and the posterior half producing the vertebral pedicle (uniting vertebral arch and centrum at the mid-dorsalventral level). By contrast, adult spinal nerves are adjacent to the posterior part of each centrum, whereas the pedicles attach anteriorly. He therefore suggested that the centrum forms by fusion of adjacent halves of two segments on each side, shifting the vertebrae half a segment caudal relative to the somites (Fig. 4).

![Fig. 4. Illustration of resegmentation.](image-url)
The polarization of somites, upon which resegmentation depends, is now well established and plays a key role in generating spinal nerve segmentation (Keynes and Stern, 1984; Kelly Kuan et al., 2004; Saga, 2012). Moreover, several lineage studies in avian embryos, using both chick-quail chimeras (Aoyama and Asamoto, 2000; Bagnalli et al., 1988; Huang et al., 1996) and retroviral labelling (Ewan and Everett, 1992), are consistent with the existence of resegmentation. These studies have also delineated sclerotome sub-regions that produce distinct vertebral elements, for example the pedicle from posterior half-sclerotome (Goldstein and Kalcheim, 1992), the annulus fibrosus from 'somitocoele' cells in the early epithelial somite (Christ et al., 2007; Huang et al., 1994) and the tendon progenitors from sclerotome cells immediately adjacent to myotome (Brent et al., 2003; Brent and Tabin, 2002). It is unlikely, however, that resegmentation involves a strict lineal correspondence between pairs of half-somites and vertebrae, as a chick study has shown that cells from one half-sclerotome can contribute to two vertebrae rather than one (Stern and Keynes, 1987). This raises the question: if the process of resegmentation is leaky in this way, how is the final periodicity established?

Whereas the notochord might play an instructive role in vertebral body periodicity in teleosts, as discussed above (Fleming et al., 2004; Grotmol et al., 2003), a recent chick embryo transplantation study supports an exclusively somite-based origin for vertebral body segmentation in this amniote species (Senthinathan et al., 2012). In amniotes, the ventral sclerotome cells on the left and right sides merge at the midline around the notochord, and their precise alignment is essential, for example to construct the segmented rings of Pax1 expression that prefigure the annulus fibrosus of the intervertebral disc (Dietrich and Gruss, 1995; Wallin et al., 1994). When left-right sclerotome pairs are experimentally misaligned (by A-P reversal of the pre-somite mesoderm on one side), the left and right half-rings of Pax1 expression also misalign segmentally at the notochord. There is no evidence, therefore, that the notochord provides instructive segmental signalling to align opposing sclerotomes (Senthinathan et al., 2012).

The role of somite polarity in amniotes

An attractive possibility is that vertebral body periodicity in amniotes is triggered by signalling interactions at the boundary between anterior and posterior halves of each somite. Cell-cell interactions at segment/compartment boundaries are well known to generate new signalling centres and cell fate diversification, for example in vertebrate phacomatoses (Kiecker and Lumsden, 2005) and Drosophila imaginal discs (Dahmann et al., 2011). Such a mechanism could generate the somitocoele cells at the early anterior-posterior boundary within chick somites, forming a signalling centre and subsequently a distinct sclerotome population ('arthrotome') that is fated to give rise to the intervertebral discs and vertebral arch joints (Mittapalli et al., 2005). Alongside the segmented expression of Pax1 noted above, the development of the amniote intervertebral disc is also known to involve the suppression of cartilage-associated gene expression via TGFβ signalling in the sclerotome (Sohn et al., 2010) and Shh expression in the notochord (Choi and Harle, 2011). However, the detailed molecular interactions that position segmental Pax1 expression and the intervertebral discs, and the status of the arthrotome in mammals, remain to be elucidated.

In support of the ‘somite polarity’ hypothesis for amniote vertebral segmentation, it is striking that knockouts of genes involved in establishing and maintaining mouse somite polarity typically show disrupted vertebral body segmentation (Table 2). The exceptions are knockouts of Mesp2 and Ripply1/2 (Takahashi et al., 2013) and of Uncx4.1 (Leitges et al., 2000; Mansouri et al., 2000), in which segmentation is disrupted at the mid-dorsoventral level (vertebral pedicles) but segmentation of the ventral sclerotome (vertebral bodies) is relatively preserved. As mouse somite polarization involves differential A-P expression of more than 750 genes (Hughes et al., 2009), this preservation could be explained by functional redundancy in the system at the ventral level, contrasting as it does with the larger number of somite polarity mutations that show disrupted ventral segmentation (Table 2).

Resegmentation and segment number in amphibians and fishes

Resegmentation has also been confirmed recently during urodele amphibian development (PiekarSKI and Olsson, 2014). It therefore operates in diverse tetrapods, and, in exploring its evolutionary origins, it will be interesting to assess its status in basal vertebrates. Indeed, somite polarization is well characterized in zebrafish (DurbIn et al., 2000; JiAng et al., 2000; Oates et al., 2005) and is evident morphologically in both teleosts and elasmobranchs (Fig. 5). In addition, a zebrafish lineage study has shown that cells from a single parent sclerotome cell can contribute to two adjacent perichordal centres (Morin-Kensicki et al., 2002). This finding shows that, as in the chick, there is no strict lineal correspondence between sclerotomes and

Table 2. Genes affecting somite polarity

<table>
<thead>
<tr>
<th>Gene</th>
<th>Somite polarity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Mouse mutations that disrupt vertebral body segmentation (ventral sclerotome)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CREB</td>
<td>P</td>
<td>(Lopez and Fan, 2013)</td>
</tr>
<tr>
<td>Δelta-1</td>
<td>P</td>
<td>(HRABé de Angelis et al., 1997)</td>
</tr>
<tr>
<td>Δelta-3</td>
<td>A</td>
<td>(Dunwoodie et al., 2002; Kusumi et al., 1998)</td>
</tr>
<tr>
<td>Hes7</td>
<td>P</td>
<td>(Bessho et al., 2001)</td>
</tr>
<tr>
<td>Lunatic Fringe</td>
<td>P</td>
<td>(Zhang and Gridley, 1998)</td>
</tr>
<tr>
<td>Meox 1/2</td>
<td>P</td>
<td>(Mankoo et al., 2003)</td>
</tr>
<tr>
<td>Paraxis</td>
<td>A+P</td>
<td>(Burgess et al., 1996; Johnson et al., 2001)</td>
</tr>
<tr>
<td>Pax3</td>
<td>A</td>
<td>(Farin et al., 2008)</td>
</tr>
<tr>
<td>Presenilin-1</td>
<td>P</td>
<td>(Shen et al., 1997; Wong et al., 1997)</td>
</tr>
<tr>
<td>Rab23</td>
<td>Not assessed</td>
<td>(Spörle and Schughart, 1998)</td>
</tr>
<tr>
<td>Tbx6</td>
<td>Presomite mesoderm</td>
<td>(Beckers et al., 2000; Nacke et al., 2000; Watabe-Rudolph et al., 2002)</td>
</tr>
<tr>
<td>Tbx18</td>
<td>A</td>
<td>(Bussen et al., 2004)</td>
</tr>
<tr>
<td>Tgfβr2</td>
<td>Intervertebral disc</td>
<td>(Baffi et al., 2006, 2004)</td>
</tr>
<tr>
<td>B: Mouse mutations that disrupt vertebral pedicle segmentation (mid-dorsoventral sclerotome)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncx4.1</td>
<td>P</td>
<td>(Leitges et al., 2000; Mansouri et al., 2000)</td>
</tr>
<tr>
<td>Mesp2</td>
<td>A</td>
<td>(Saga et al., 1997; Takahashi et al., 2013)</td>
</tr>
<tr>
<td>Ripply 1/2</td>
<td>A</td>
<td>(Morimoto et al., 2007; Takahashi et al., 2013)</td>
</tr>
</tbody>
</table>
Vertebral segmentation and resegmentation

The third issue we have addressed is the still widely debated question of how the mature segmental pattern is established. In teleosts, it is striking that the site of union between vertebral arches and centra is variable, even within a single animal, whereas the
arches are consistently intersegmental (Farugi, 1935; Lauder, 1980). This might be aided by the relative independence of telost arch and chordacentrum development already noted, which perhaps promotes oscillatory swimming movements (Lauder, 1980). Enhanced axial flexibility might also explain the evolution of diplopondyly in elasmobranchs and holocephalans, where the ratio of centra to arches is 2:1 rather than 1:1; and, conversely, axial stabilization might be promoted where the ratio is 1:2 (Maxwell et al., 2013).

Such remarkable diversity of centrum segmental patterning in telosts has been replaced in tetrapods by a resegmentation system with greater fixity. A plausible scenario is that it evolved in the presence of somite polarity, under the regulation of the somite clock (Dias et al., 2014), so facilitating construction of diverse types of centra from scleromite sub-components. The amniote centrum typically develops from a single ossification centre, but that of stem tetrapods comprises two alternating anterovertebral (A-V) and posterodorsal (P-D) components, creating so-called ‘rachitomous’ vertebrae. These components can also combine in reverse order along the A-P axis (P-D→A-V rather than A-V→P-D), as recently described for Ichthyostega, and this arrangement might represent the ancestral tetrapod condition (Pierce et al., 2013).

We should also point out that the precise functional advantage of resegmentation in amniotes remains unclear. After Remak’s anatomical observations, von Ebner (1888) suggested that, as axial myotome derivatives retain their original segmental positions, scleromite resegmentation allows axial muscles to straddle the intervertebral joints and so promote trunk flexibility. Although this is the preferred explanation in contemporary textbooks, there is no evidence for segmental muscles that straddle neighbouring centra in representative reptiles, birds and mammals, including humans (Baur, 1969). It might apply in humans to certain deep segmental back muscles, the attachment of which is solely to dorsal vertebral arch elements. Here, resegmentation would shift the registration between these dorsal muscles and the ventral intervertebral disc joints, perhaps allowing the muscles to influence the joints with greater mechanical advantage. But it is equally plausible that von Ebner’s functional explanation, although elegant, is an oversimplification. Further exploration of the structure-function relationships of the vertebral-muscular system in fish, and how these differ in tetrapods, would shed light on the evolutionary origins of this functional advantage, although it is equally plausible that von Ebner’s functional explanation is the result of natural selection for greater locomotor flexibility.

Future challenges

A key unanswered question is how the uniformity of segmentation is established in the adult vertebral column in the absence of a strict lineal correspondence with somites. In amniotes, we have highlighted the possible role of a signalling centre established at the A-P boundary within each scleromite. In telosts, there is evidence that chordacentrum segmentation might arise independently of somite segmentation, and several studies have indicated a role for the telost notochord in establishing the segmental pattern. While at present it is conceptually difficult to understand how such an overtly nonsegmented structure might impose this, an A-P periodicity in the notochord cell cycle has been reported recently (Sugiyama et al., 2014).

Much of the recent experimental evidence for the cellular or even evolutionary origins of different vertebral components presented here has come from gene expression studies or the use of single gene markers to identify cells of a particular lineage. As highlighted in Table 1, there are questions about the reliability of such markers and it is important to bear in mind that very few genes are lineage specific. We therefore urge caution in the interpretation of lineage relationships based solely on gene expression, and suggest that the definitive relationship between tissues and structures can only be elucidated by detailed lineage tracing studies. The growing repertoire of techniques for lineage tracing in vivo (such as photoconvertible fluorescent proteins, cre-lox labelling technologies and inducible gene expression) offers huge potential to address these questions with a cleaner experimental approach than previous studies using grafting or dye labelling. Simultaneously, a deeper knowledge of the molecular mechanisms of scleromite and vertebral development in fish, including elasmobranchs and agnathans, and the degree to which these are conserved in amniotes, will be essential to appreciate how the astonishing diversity of vertebral patterning has evolved.

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