REVIEW

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); Oates et al. (2012)]. During development, each somite differentiates into the dermomyotome, which forms dermis and

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skeletal muscle, and the sclerotome, which gives rise to vertebral components and their associated joints. Our understanding of sclerotome formation comes mostly from studies in mouse and chick, which show that sclerotome development is initiated when sonic hedgehog (Shh) (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994) and the bone morphogenetic protein (Bmp) antagonists noggin and gremlin (Hirsinger et al., 1997; McMahon et al., 1998; Stafford et al., 2011) signal from the midline notochord to induce adjacent somite cells to undergo an epithelial-to-mesenchyme transition and adopt a sclerotome fate (Christ et al., 2004). The sclerotome expresses transcription factors, such as *Pax1*, *Pax9* and *Twist*, which are used as sclerotome markers (Table 1).

In amniote embryos, the medially positioned sclerotome forms a substantial proportion of the somite. During development, its cells migrate to surround the midline notochord and neural tube. The cells differentiate first into chondrocytes that deposit a cartilage intermediate, and then into osteoblasts that replace the cartilage with bone (a process known as endochondral ossification, see Box 2) to form the centra (ventral, around the notochord) and the vertebral arches (dorsal, around the spinal cord) (Chal and Pourquié, 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 craniate

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
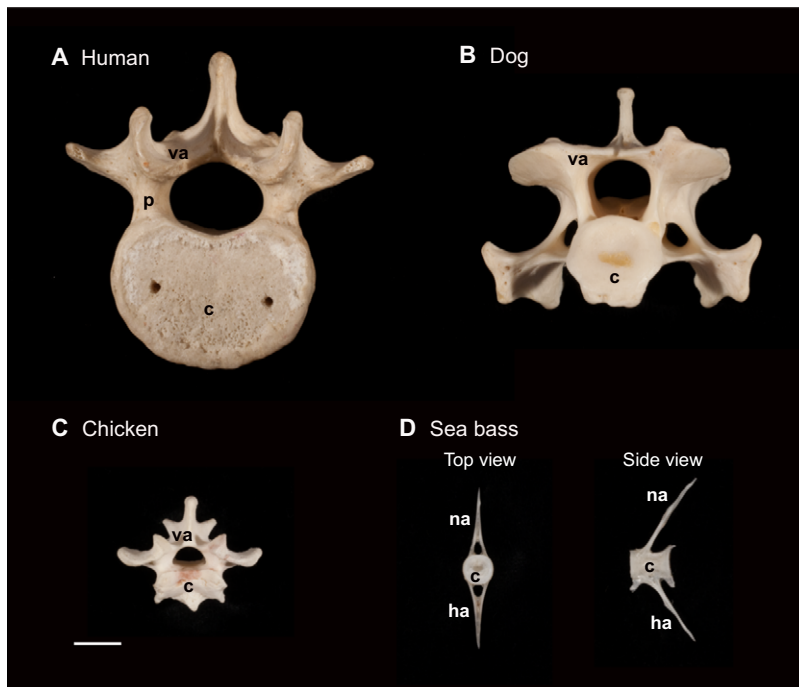
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Fig. 1. Diversity in the structure of vertebrae between different vertebrate lineages. Vertebrae from animals of different vertebrate lineages show remarkable diversity in their three-dimensional shape, as illustrated by images of a human thoracic vertebra (A), a dog cervical vertebra (B), a chicken thoracic vertebra (C), and a sea bass thoracic vertebra (D). Vertebrae consist of a centrum (c) and vertebral arches (va). Vertebral arches can be further defined as either neural arches (na) that project dorsally around the spinal cord or haemal arches (ha) that project ventrally. The pedicle (p), a named feature of amniote vertebrae, lies at the base of the neural arch, where it joins the centrum. Each vertebra is imaged to show its anterior aspect, dorsal above and ventral below, with the exception of the sea bass vertebra, which is also imaged at side view (dorsal above, ventral below). Note the relative size of the centrum to the arches. All vertebrae are shown at the same magnification; scale bar: 1 cm.

agnathan group, the hagfish, were thought to have no vertebrae; hence the formal classification of Vertebrata as a subdivision of the Craniata that excludes hagfish. However, a forgotten study from the end of the 19th century identified minute cartilaginous nodules positioned ventral to the notochord in the tail of some hagfish species (Ayers and Jackson, 1901). Recent reinvestigation has revealed that these structures share molecular developmental features with the vertebral elements of other vertebrates and are associated with cells that express orthologues of the sclerotome markers *Pax1/9* and *Twist* (Ota et al., 2011, 2013). Although

showing no clear segmental organization, these structures are reminiscent of haemal arches (Ota et al., 2011; defined in Box 1) and have the histological appearance of cartilage, like lamprey and elasmobranch haemal arches.

Analysis of fossil groups has shed further light on the evolutionary origin of vertebral elements and their segmentation. The mostly soft-bodied fossil *Haikouichthys*, described as a stem craniate, has elements positioned along its notochord that might represent vertebral rudiments (Shu et al., 2003). Furthermore, the ancient agnathan *Euphanerops* shows mineralized vertebral elements, and among these might be morphologically distinguishable dorsal and ventral vertebral arch elements (Janvier and Arsenault, 2002). Their distribution is clearly periodic along the axis, resembling the lamprey dorsal arch elements (Janvier, 2007; Janvier and Arsenault, 2002). The phylogenetic position of *Euphanerops* is not clear, but it could represent a stem cyclostome (Janvier, 2011). It has been proposed by Ota and colleagues (2011, 2013) that the presence of only dorsal elements in the lamprey and ventral elements in the hagfish represents evolutionary losses of an ancestral condition in which both dorsal and ventral elements were present, matching the condition observed in gnathostomes. The discovery and characterization of *Euphanerops* vertebral arch elements supports this hypothesis. The molecular and histological evidence in extant agnathans and fossil examples of stem craniates/agnathans points, therefore, towards an early evolution of the vertebral arches, before and independent of the vertebral bodies. This would imply that vertebral elements are present in all of the craniate groups and that reconsideration of the Craniata/Vertebrata hierarchy is warranted (Janvier, 2011; Ota et al., 2011, 2013).

Centra arose within gnathostomes

Mineralized centra are present in almost all of the major lineages of Eugnathostoma, a group comprising all jawed vertebrates except placoderms (Fig. 2). The three major living gnathostome groups – chondrichthyans, 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

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 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 acanthodians (Miles, 1970) and osteichthyans (Schaeffer, 1967), and particularly the dipnoans (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.

Table 1. Genes used as markers of sclerotome and osteoblast differentiation

Gene	Caveats	References
Sclerotome markers		
Twist	Chick/mouse – not restricted to the sclerotome lineage and expressed in other tissues in the trunk (neural crest, trunk mesoderm) Zebrafish – 2 orthologues, *Twist1a* and *Twist1b*, expressed in separate domains Medaka – sclerotome and 'putative' neural crest	(Barnes and Firulli, 2009) (Yeo et al., 2009) (Yasutake et al., 2004)
Pax9	Mouse – expression restricted to the dorsal half of sclerotome and in non-somitic tissues Teleosts – expression throughout the sclerotome	(Peters et al., 1999) (Peters et al., 1998) (Mise et al., 2008) (Fleming et al., 2004) (Chatterjee et al., 2011)
Pax1	Chick – early marker of ventral sclerotome Medaka – general sclerotome marker Zebrafish – 2 orthologues, *Pax1a* and *Pax1b*[a]	(Christ et al., 2004) (Mise et al., 2008) ([a]http://zfin.org)
Foxc2 (*Mfh1, cFkh1*)	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* No homologue identified in teleosts[b]	(Christ et al., 2004) ([b]based on homology information in Homologene database: http://www.ncbi.nlm.nih.gov/homologene)
Scleraxis	Mouse and chick – expressed in part of sclerotome that gives rise to tendons and ligaments Zebrafish possess 2 orthologues	(Perez et al., 2003) (Christ et al., 2004)
Zic1	Chick – sclerotome expression but also nascent myotome and non-migratory neural crest Mouse – dorsal sclerotome and dermomyotome *Xenopus* – neural crest development Zebrafish – dorsal somite, partially overlapping with myotome Medaka – dorsal somite	(Sun Rhodes and Merzdorf, 2006) (Aruga et al., 1999) (Rohr et al., 1999) (Moriyama et al., 2012)
Tbx18	Mouse – initially anterior half-somite, then just sclerotome and non-somitic domains Zebrafish – initially anterior half somite, then around horizontal myoseptum	(Kraus et al., 2001)
Differentiation markers of sclerotome/chondrocytes/osteoblasts		
Sox9	– Chondrocyte marker, including those derived from cranial neural crest – Expressed in the notochord in mouse and zebrafish – 2 orthologues in zebrafish	(Mori-Akiyama et al., 2003) (Barrionuevo et al., 2006) (Yan et al., 2005)
Col2a	– Similar expression to *Sox9*	(Zhao et al., 1997)
Col10a1	Mouse – chondrocytes Zebrafish – in osteoblasts of bones formed by direct ossification Medaka – in some of sclerotome-derived but also expressed in other vertebrae-forming cells of unknown origin	(Avaron et al., 2006) (Renn et al., 2013)
Sp7 (*Osterix*)	– Early osteoblast marker	(Nakashima et al., 2002) (Li et al., 2009)

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.

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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 teleosts.

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 chondroid 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).

Centra subtypes and their formation: insights into the evolution of vertebrae

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
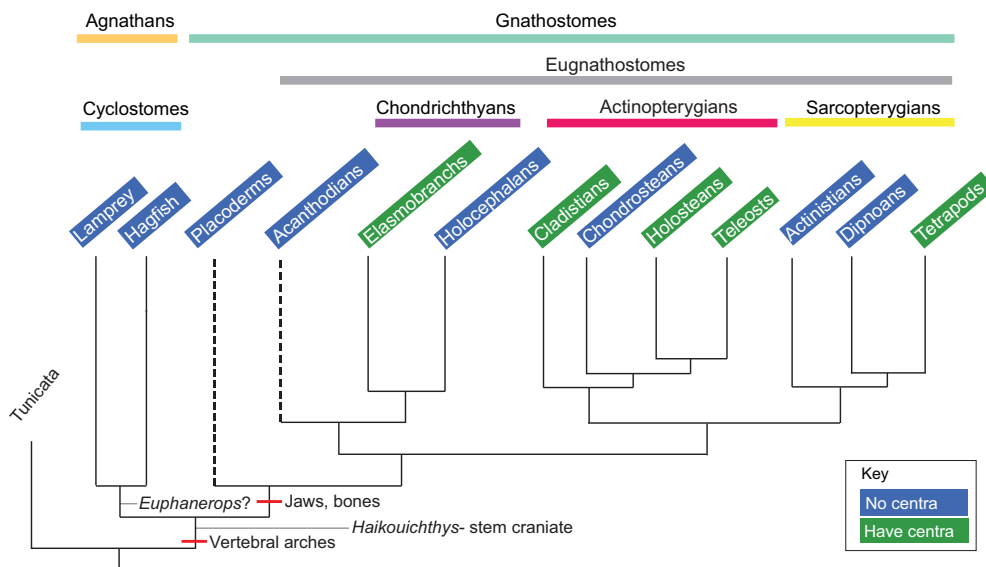

Fig. 2. The phylogeny and appearance of vertebral elements in vertebrate phyla. Vertebral arches are thought to have arisen in the stem group that gave rise to agnathans and gnathostomes, whereas centra appear later and are only present in gnathostomes. Mineralized vertebral elements have been identified 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 anamniotes (fishes and amphibians).

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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.

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 perichordal centrum (Ekanayake and Hall, 1988; Nordvik et al., 2005). Whereas the cells that secrete the perichordal 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,
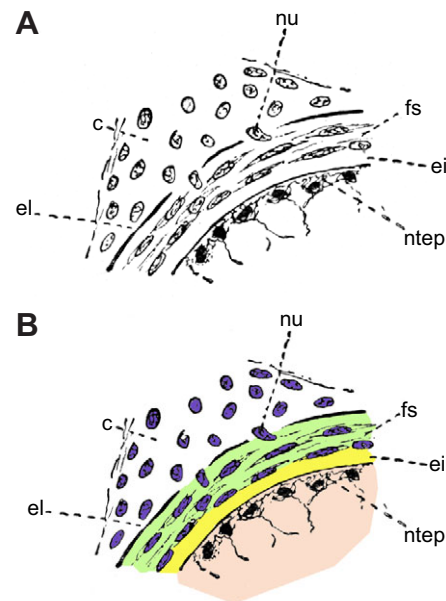

Fig. 3. Invasion of the notochord sheath by somite-derived cells in an elasmobranch. (A) Drawing of transverse section of the notochord of a *Scyllium canicula* (lesser spotted dogfish) embryo showing a sector of the notochord sheath, comprising the inner epithelial 'chordablast' layer (labelled ntep), surrounded by the 'elastica interna' or inner layer of the fibrous sheath (ei), and the fibrous sheath (fs) which is invaded by sclerotome cells.
A nucleated cell (nu) is shown perforating the elastica externa (el) at the outer part of the fibrous sheath. Cartilage (c) is present in the sclerotome outside the sheath. The discrete layers and cell types are more apparent in the false-coloured image (B) in which sclerotome cells are coloured purple, the fibrous sheath is green, the elastic externa is pale yellow and the chordoblasts are pink. Drawing reproduced from figure 9B in: E. S. Goodrich, *Studies on the Structure and Development of Vertebrates*, 1930, Macmillan and Co., London; reproduced with permission of Palgrave Macmillan.

osteoblasts identified by the expression of *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 *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 *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 *sp7⁻* cell population expressing *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 *twist* in medaka results in aberrant vertebral arches but normal centrum development (Yasutake et al., 2004). Likewise in single and double knockdowns of *pax1* and *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 *ex vivo* culture secrete an osteoid matrix, and laser ablation of single notochord cells *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).

Segmental patterning

The evidence reviewed above indicates that the mechanisms building teleost and tetrapod vertebrae differ significantly. Whereas tetrapod centra are typically described as perichordal, there is a striking duality in the origins of the teleost centrum: it forms from the primary ossification of the chordacentrum within the notochordal sheath, followed by reinforcement perichordal ossification by sclerotome cells, and it is likely that two distinct osteoblast populations, one of them derived from the notochord, cooperate to form the complete centrum (Spoorendonk et al., 2008). This implies that the teleost sclerotome might play a secondary role in centrum development, perhaps maintaining rather than initiating the segmental pattern. Whereas tetrapod centrum patterning is instructed by somite patterning and polarization, the notochord appears to play more of an instructive role in teleost centrum patterning.

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 protovertebrate *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, *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-dorsoventral 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).
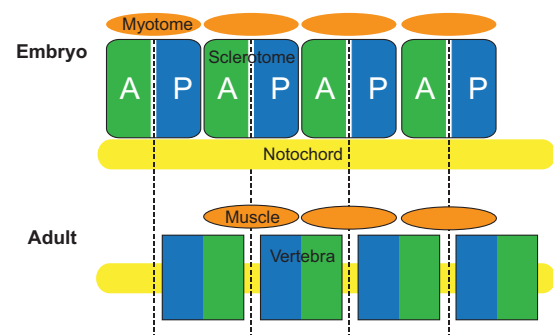

Fig. 4. Illustration of resegmentation. Following differentiation of the somite, each sclerotome is divided into anterior (A) and posterior (P) halves, and each A/P pair lies in register with the myotome derived from the same somite. During resegmentation, the vertebral bodies form by the recombination of neighbouring sclerotome halves from adjacent segments, causing a half-segment shift in the registration of embryonic sclerotomes compared with adult centra (dashed lines). As the myotomes retain their original segmental positions, they are hypothesized to straddle the intervertebral discs, promoting trunk flexibility (von Ebner, 1888).

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; Bagnall 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

rhombomeres (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 Harfe, 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 amphibia 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 centra (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

| Gene	Somite polarity	References
A: Mouse mutations that disrupt vertebral body segmentation (ventral sclerotome)		
CREB	P	(Lopez and Fan, 2013)
Delta-1	P	(Hrabě de Angelis et al., 1997)
Delta-3	A	(Dunwoodie et al., 2002; Kusumi et al., 1998)
Hes7	P	(Bessho et al., 2001)
Lunatic Fringe	P	(Zhang and Gridley, 1998)
Meox 1/2	P	(Mankoo et al., 2003)
Paraxis	A+P	(Burgess et al., 1996; Johnson et al., 2001)
Pax3	A	(Farin et al., 2008)
Presenilin-1	P	(Shen et al., 1997; Wong et al., 1997)
Rab23	Not assessed	(Spörle and Schughart, 1998)
Tbx6	Presomite mesoderm	(Beckers et al., 2000; Nacke et al., 2000; Watabe-Rudolph et al., 2002)
Tbx18	A	(Bussen et al., 2004)
Tgfbr2	Intervertebral disc	(Baffi et al., 2006, 2004)
B: Mouse mutations that disrupt vertebral pedicle segmentation (mid-dorsoventral sclerotome)		
Uncx4.1	P	(Leitges et al., 2000; Mansouri et al., 2000)
Mesp2	A	(Saga et al., 1997; Takahashi et al., 2013)
Ripply 1/2	A	(Morimoto et al., 2007; Takahashi et al., 2013)
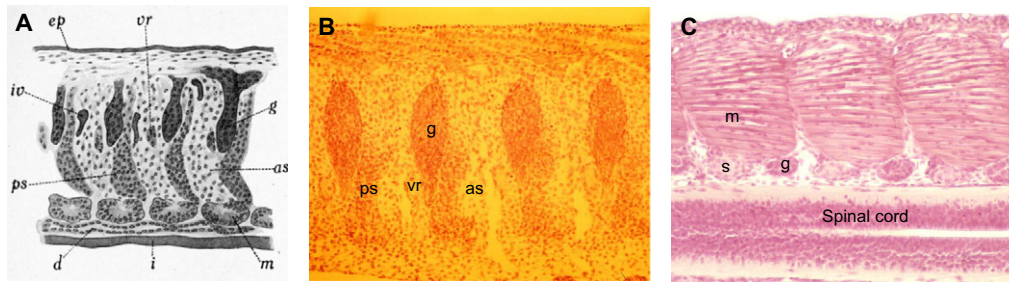
Fig. 5. Somite polarity in elasmobranch and teleost fish. (A) Sagittal section of a *Scyllium canicula* (elasmobranch/lesser spotted dogfish) embryo showing the sclerotome polarized into anterior (*as*) and posterior (*ps*) halves. The ventral root (*vr*) of the spinal nerve lies in the anterior half-sclerotome; *g*, sensory ganglion; *ep*, epidermis; *iv*, intersegmental vein; *d*, mesonephric duct; *m*, mesonephric tubule; *i*, intestinal wall. Drawing reproduced from figure 16C in: E. S. Goodrich, *Studies on the Structure and Development of Vertebrates*, 1930, Macmillan and Co., London; reproduced with permission of Palgrave Macmillan. (B) Histological section of a *Squalus acanthias* (elasmobranch/spiny dogfish) embryo. The main features in this sagittal section closely match Goodrich's drawing (panel A); the sclerotome is polarized into anterior (as) and posterior (ps) halves, and the ventral root (vr) lies in the anterior half-sclerotome; g, sensory ganglion. Image reproduced courtesy of the Department of Zoology collection, University of Cambridge, UK. (C) Longitudinal section of larval *Salmo trutta* (teleost/brown trout). The section shows somite derivatives on one side of the spinal cord. Each segment contains a dorsal root ganglion (g) and myotome (m); the sclerotome is condensed in the posterior (left) part of each segment (s), separated from the ganglion in the anterior (right) part of each segment. Image reproduced courtesy of the Department of Zoology collection, University of Cambridge, UK, and published previously as figure 4 in Keynes and Stern (1988).

vertebrae. However, unlike amniotes, the teleost centrum lies within each muscle segment, negating the requirement for a resegmentation frameshift (Lauder, 1980).

The observation that there is no 1:1 relationship between somites and vertebrae raises the question as to what determines the number of vertebral segments. Teleost vertebrae can form with normal segmental periodicity when somite patterning is disrupted, as in the *tbx6/fused somite* mutant (Fleming et al., 2004; Nikaido et al., 2002; van Eeden et al., 1996). In addition, centrum segmentation might be lost in situations in which normal somite patterning is maintained, for example in the over-ossification observed in *cyp26b* and *enpp1* mutants (Apschner et al., 2014; Laue et al., 2008; Spoorendonk et al., 2008). Disruption of the segmentation clock in zebrafish *hes6* mutants results in fewer somites and, later in development, fewer vertebrae (Schröter and Oates, 2010), suggesting that vertebral segment number is indeed linked to somite number. However, an alternative possibility is that any somite-independent mechanisms that may exist could also be segmented by the 'clock'. The observation that periodic cell cycle transitions occur within the notochord at twice the periodicity of somite segmentation provides some evidence not only for an intrinsic segmental pattern within the notochord but also a link to somitic segmentation (Sugiyama et al., 2014).

Conclusions
Vertebral arches and centra are independent units
Our intention here has been to combine evidence from the fossil record (often overlooked in developmental biology studies) with recent experimental evidence in model organisms. The first point to highlight is that arches and centra do not appear coincidentally in the fossil record. Arches are present in agnathans and precede the appearance of centra. Recent developmental and molecular studies in lampreys and hagfish have greatly enhanced our understanding of vertebral development in jawless fishes. However, we note that questions of sclerotomal origins, morphology and morphogenesis either have never been studied, or, for lamprey, have not been critically examined during the past 100 or more years. Developmental studies have largely focused on the vertebrae (arches and centra) as a whole, assuming they form as a single unit, but as they appear at different times in the fossil record we suggest they should be considered independent elements. Moreover, the cells giving rise to

these elements, their patterning and the modes of mineralization might well be different.

Homology between centra
As the fossil evidence for the appearance of centra is patchier than for arches, the second question raised by this Review is whether centra are homologous structures among gnathostomes, or even eugnathostomes. This patchiness might in part be due to the cartilaginous composition of such elements in some species, which would not fossilize, and mineralized centra in others. Centra, or parts of them, sometimes form in association with the vertebral arches, but they are essentially always in association with the notochord. Another key issue that we have considered here is that in many species within two major groups of fish, elasmobranchs and teleosts, the chordacentrum mineralizes within the sheath of the notochord itself. The origin of cells mineralizing the chordacentra could well vary among species, as we have considered in some detail. By contrast, it is likely that perichordal centra and arches always develop from sclerotome.

As we have detailed here, chordacentra are also becoming very well known in teleosts. Arratia et al. argue strongly that elasmobranch and teleost chordacentra are not homologous, primarily because of lack of connecting intermediates (Arratia et al., 2001), but we would argue that there are marked similarities between the chordacentra of the two groups. The differences are intriguing and might help us to understand evolutionary divergence, so we want to encourage further comparative research. We also propose that mineralization of both cartilage and bone is evolutionarily labile, and suggest that homology of perichordal centra within gnathostomes be neither rejected nor accepted at present (Box 3). The hypothesis that chordacentra might be homologous among at least crown gnathostomes (i.e. including both chondrichthyans and osteichthyans) is admittedly more tentative, but is offered in the spirit of encouraging new studies that continue to address these interesting issues.

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 teleost arch and chordacentrum development already noted, which perhaps promotes oscillatory swimming movements (Lauder, 1980). Enhanced axial flexibility might also explain the evolution of diplospondyly 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 teleosts 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 sclerotome sub-components. The amniote centrum typically develops from a single ossification centre, but that of stem tetrapods comprises two alternating anteroventral (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, sclerotome 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 vertebra-muscular system in fish, and how these differ in tetrapods, would shed light on the evolutionary origins of resegmentation.

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 sclerotome. In teleosts, there is evidence that chordacentrum segmentation might arise independently of somite segmentation, and several studies have indicated a role for the teleost notochord in establishing the segmental pattern. While at present it is conceptually difficult to understand how such an overtly non-segmented 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 sclerotome 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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