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Early Evolution of Vertebrate Skeletal Tissues and Cellular Interactions, and the Canalization of Skeletal Development

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ABSTRACT The stratigraphically earliest and the most primitive examples of vertebrate skeletal mineralization belong to lineages that are entirely extinct. Therefore, palaeontology offers a singular opportunity to address the patterns and mechanisms of evolution in the vertebrate mineralized skeleton. We test the two leading hypotheses for the emergence of the four skeletal tissue types (bone, dentine, enamel, cartilage) that define the present state of skeletal tissue diversity in vertebrates. Although primitive vertebrate skeletons demonstrate a broad range of tissues that are difficult to classify, the first hypothesis maintains that the four skeletal tissue types emerged early in vertebrate phylogeny and that the full spectrum of vertebrate skeletal tissue diversity is explained by the traditional classification system. The opposing hypothesis suggests that the early evolution of the mineralized vertebrate skeleton was a time of plasticity and that the four tissue types did not emerge until later. On the basis of a considerable, and expanding, palaeontological dataset, we track the stratigraphic and phylogenetic histories of vertebrate skeletal tissues. With a cladistic perspective, we present findings that differ substantially from long-standing models of tissue evolution. Despite a greater diversity of skeletal tissues early in vertebrate phylogeny, our synthesis finds that bone, dentine, enamel and cartilage do appear to account for the full extent of this variation and do appear to be fundamentally distinct from their first inceptions, although why a higher diversity of tissue structural grades exists within these types early in vertebrate phylogeny is a question that remains to be addressed. Citing recent evidence that presents a correlation between duplication events in secretory calcium-binding phosphoproteins (SCPPs) and the structural complexity of mineralized tissues, we suggest that the high diversity of skeletal tissues early in vertebrate phylogeny may result from a low diversity of SCPPs and a corresponding lack of constraints on the mineralization of these tissues. *J. Exp. Zool. (Mol. Dev. Evol.) 306B, 2006.*

The study of fossilized skeletal tissues in early vertebrates has a long tradition, dating back at least to Agassiz (1833–43). Skeletal tissues are mineralized and, therefore, readily fossilized, and preserve a considerable amount of developmental data in the form of growth lines, tissue topologies and cell polarities. Thus, interest in palaeohistology emerged because these data are readily reconcilable with knowledge of developmental processes as revealed by experimental analysis of living vertebrates. This is particularly fortunate because living vertebrates provide little insight into the evolution of the skeleton and of skeletal tissues. The most primitive living vertebrates with mineralized skeletal tissues, the sharks and primitive bony fishes, possess a skeleton that is, aside from the detail, akin to our own. Meanwhile, the most primitive of all living vertebrates, the hagfishes and lampreys, lack any mineralized skeletal tissues (but see Bardack and Zangerl,

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'71; Langille and Hall, '93), with little more than some simple cartilaginous elements to support the brain, gills pouches, tail fin. The phylogenetic position of the earliest skeletonizing vertebrates intercalates precisely these two grades of skeletonization, and, from their remains, it is possible to unravel the gradual assembly of the skeletal systems that are generally considered characteristically vertebrate (Donoghue and Sansom, 2002). Thus, although the fashion for integrative approaches to understanding developmental evolution has emerged as a relatively recent phenomenon, this has been the main modus operandi for palaeohistology from its inception.

Despite early interest, attempts to synthesize palaeohistological data to provide a comprehensive understanding of early skeletal evolution did not begin until the latter half of the twentieth century (Moss, '64; Ørvig, '67; Halstead, '87; Smith and Hall, '90), coincident with a renaissance of interest in developmental evolution within experimental embryology. From these syntheses, two conflicting conclusions have emerged that have very different implications for models of the emergence of skeletal tissues and systems within vertebrates. Firstly, although there has been much debate over the timing and phylogenetic appearance of tissue types and tissue grades, it has been concluded that the universe of skeletal tissues was established early within vertebrate phylogeny (Moss, '64; Ørvig, '67; Halstead, '74; Reif, '82; Maisey, '88; Smith and Hall, '90). Secondly, some authors (sometimes even the same authors) have insinuated that this conclusion may be an artefact of a tradition of assigning fossil tissues to categories established on the basis of more derived, living vertebrates (Halstead, '87). In this view, skeletal tissues are at first more homogenous and plastic, reflecting a precursor stage to the establishment of the discrete tissue types that emerged later in vertebrate phylogeny: bone, dentine, enamel and cartilage. Thus, there appears to be a spectrum of variation both within (Sansom et al., '94; Smith and Sansom, 2000) and between tissues types that, in living vertebrates, are derived from distinct cell lineages or germ layers (Ørvig, '51, '58, '67; Denison, '63; Smith and Hall, '90; Sansom et al., '94; Smith and Sansom, 2000; Hall, 2005).

These opposing views provide very different perspectives on the appearance of skeletal tissues and, by inference, the manner in which developmental systems are established. However, these perspectives are based on interpretations of palaeohistological data that are subject to one or more limiting biases: an emphasis on the changing patterns with respect to time, rather than to phylogeny, and/or a focus on raw tissue grades, rather than on evolution with respect to the skeletal systems within which they are manifest. We have previously teased apart these biases in an attempt to unravel the assembly of the vertebrate skeleton with respect to its distinct embryological components (Donoghue and Sansom, 2002). It is our current aim to examine the evolution of tissue types within these skeletal systems. We will begin by considering the composition of the vertebrate skeleton and the evolutionary relationships of living and extinct primitive vertebrates. This will provide a framework within which to examine evidence for the evolution of skeletal tissues within early vertebrate phylogeny. On this basis, we will consider the two opposing models concerning the emergence of vertebrate skeletal tissues.

SKELETAL SYSTEMS AND EVOLUTIONARY RELATIONSHIPS

Before we progress further, it is important that we consider the fact that the skeleton is an embryological and phylogenetic composite (Donoghue and Sansom, 2002). Thus, it would be inappropriate to conflate tissue data between these skeletal systems. The vertebrate skeleton is composed of a dermoskeleton and endoskeleton, the latter being a composite of viscerocranium, neurocranium, axial and appendicular skeletons. Although the dermoskeleton and viscerocranium are dermally and endodermally derived, respectively, they are united by the fact that they are also neural crest derived, a factor that may well transcend the significance of their epithelial origin (Hall, '98). Meanwhile, the remainder of the endoskeleton, including the neurocranium, axial and appendicular skeletons (i.e., the viscerocranium aside), is mesodermally derived.

Given our aim of deciphering the manner in which characteristic skeletal tissues emerged during early vertebrate phylogeny, it is necessary at the outset to establish the evolutionary relationships of the living and extinct groups from which data are to be considered. Thus, it will be possible to establish the evolutionary appearance of characters, without recourse to orthogenetic trends or patterns of fossil appearance within the geological record, both of which are problematic in their own rights.

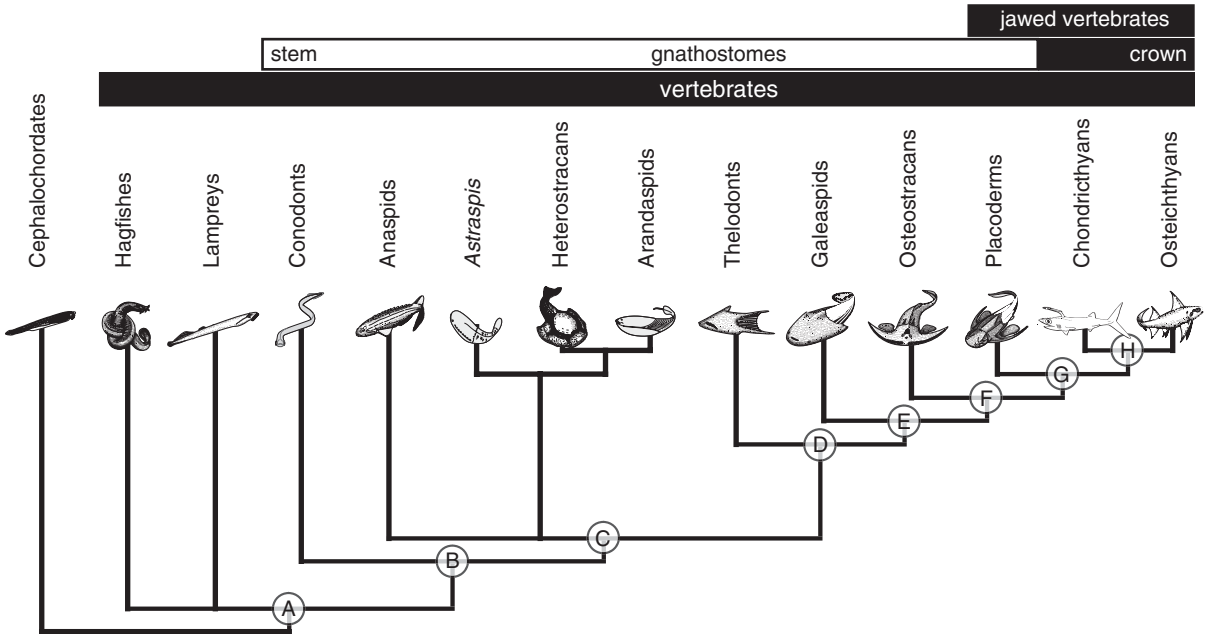

Fig. 1. Phylogeny of vertebrates adopted in this paper, based on the phylogenetic analyses of Donoghue and Smith (2001). Note the distinction between gnathostomes and jawed vertebrates. The letters (A–H) denote nodes at which significant steps in vertebrate skeletal evolution occurred, as inferred through ACCTRAN optimization of data from the terminal taxa. (**A**) Origin of a vertebrate skeleton including a notochordal sheath, fin rays, neurocranium and viscerocranium, though entirely composed of unmineralized cartilage. (**B**) Origin of a mineralized skeleton, dentine and enamel comprising the odontode developmental module, first manifest in the viscerocranium. (**C**) Origin of a mineralized dermoskeleton composed of odontodes supported by extensively developed bone, imposing mineralization upon the collagenous layers of the dermis. (**D**) Odontodes associated with the viscerocranium, including either the gill arches or the nasohypophyseal openings. (**E**) Origin of a mineralized neurocranium composed of globular calcified cartilage. (**F**) Mineralized neurocranium encompassing equivalents of the scaula and coracoid, as well as the pericardial region, composed of cellular perichondral bone; cellular dermal bone also first encountered in the dermoskeleton. (**G**) Mineralized viscerocranium, axial skeleton, appendicular skeleton and fin radials. (**H**) Endochondral bone.

It is right and proper that the evolutionary relationships of extinct organisms should be considered within a framework of relationships already established on the basis of their living relatives, because the latter are better known, or at least have the potential to be better known (Hennig, ’81; Donoghue, 2005). However, the evolutionary relationships of the jawless hagfishes and lampreys and the jawed vertebrates (chondrichthyans and osteichthyans) have been the subject of open debate since the late nineteenth century (Janvier, ’96a), and remain so today (Furlong and Holland, 2002a). Fortunately, when it comes to understanding early skeletal evolution, little depends upon whether hagfish and lampreys are more closely related to one another (Delarbre et al., 2002), or whether lampreys are more closely related to jawed vertebrates (Løvtrup, ’77). More critical is the question of how the many extinct clades of bony jawless vertebrates relate to living vertebrates; these clades have hitherto been closely allied with their living jawless counterparts (Stensiö, ’68; Halstead, ’82) and, by degrees of relationship, with living jawed vertebrates (Janvier, ’81). Over the past two decades, a near-universal consensus has been reached in favour of the paraphyly of the extinct bony jawless vertebrates (Forey, ’84, ’95; Forey and Janvier, ’93, ’94; Janvier, ’96a,b, ’98; Donoghue et al., 2000, 2003; Donoghue and Smith, 2001; Sansom et al., 2005a). Details of precise relationship have shown differences, though even in this respect, consensus has begun to emerge, with only the affinities of anaspids and the enigmatic *Eriptychius* differing between the most recent analyses. In the following, we adopt the phylogenetic scheme of Donoghue and Smith (2001) and note these equivocations (Fig. 1).

THE DERMOSKELETON

The most primitive vertebrate skeleton is undoubtedly the viscerocranium, but the dermoskeleton is first to show mineralization within

vertebrate phylogeny (Donoghue and Sansom, 2002). Although there are older records (Smith et al., '96b; Erdtmann et al., 2000), the earliest unequivocal record of a dermoskeleton is composed of the stereotypical suite of tissues seen in more derived relatives: composites of dentine, enameloid and bone (Sansom et al., 2005a), indicating plesiomorphy of the odontode unit of development, first seen within the dermoskeleton and later within the viscerocranium in the form of teeth. However, the structure of these tissues is not so characteristic, and there has been considerable debate over whether some of these tissues are in fact intermediate between some of the classical tissues types (Ørvig, '51, '58, '67; Denison, '63; Smith and Hall, '90; Sansom et al., '94; Smith and Sansom, 2000; Hall, 2005), perhaps betraying a common evolutionary origin that is no longer apparent from the embryology and histology of living representatives. Below, we examine the evolution of these tissue types from their most plesiomorphic manifestations through to better-understood living representatives.

BONE EVOLUTION

The evolution of bone has been the subject of endless debate, centred especially around the relative plesiomorphy of cellularity and acellularity in the dermoskeleton (Denison, '63; Halstead

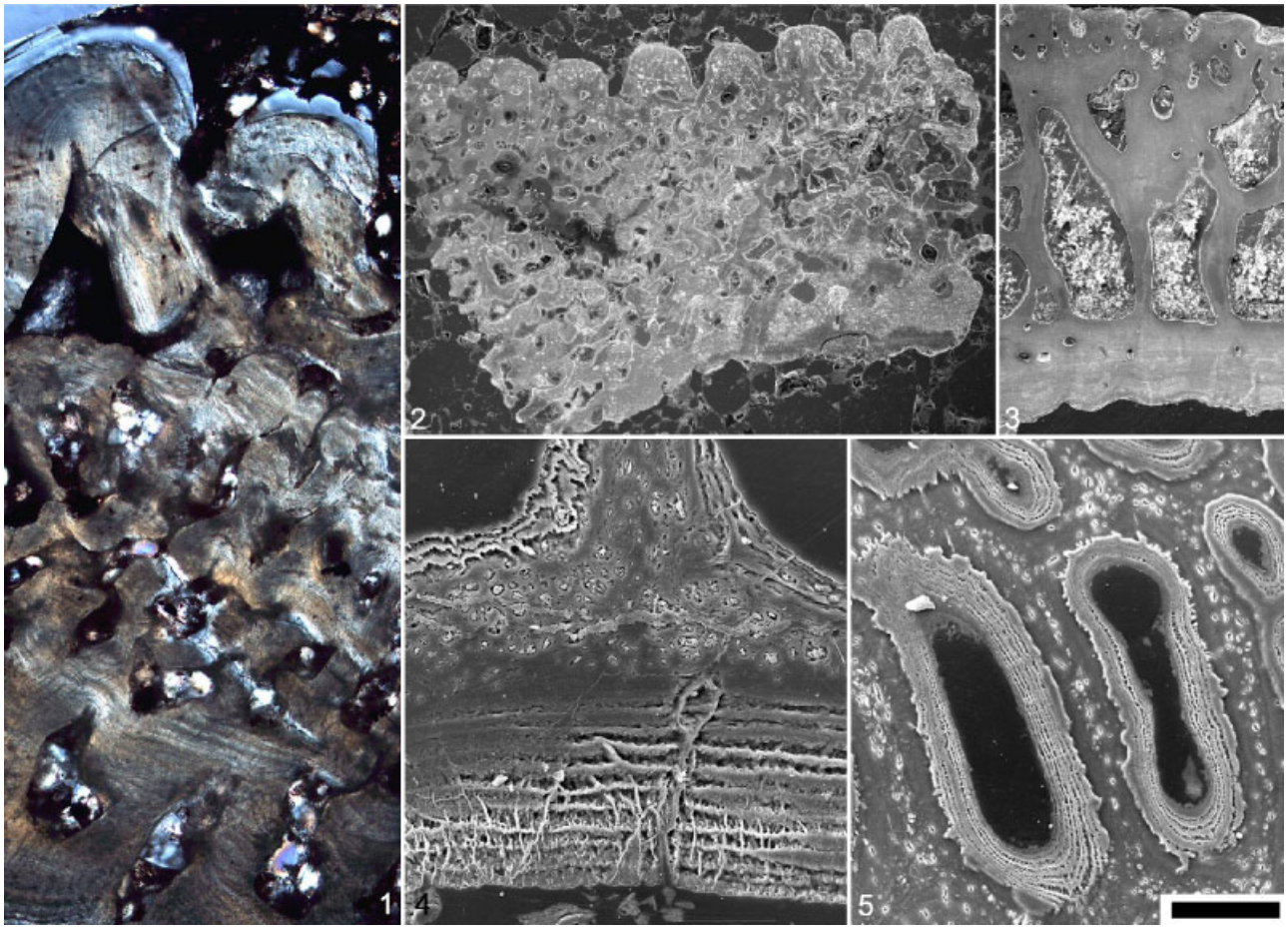

Fig. 2. Dermal skeletal structure of pteraspidomorphs. (**1**) Nomarski interference optical micrograph through the dermal armour of the Ordovician *Astraspis*; note the fine calibre dentine tubercles capped by moncrystalline enameloid, the spongy ''aspidin'' comprising the middle layer and the lamellar basal layer (BU 4471). (**2**) Electron micrograph of an etched specimen of *Eriptychius* illustrating the spongy nature of the dermal armour and surmounting coarse calibre dentine tubercles (BU 4472). (**3**) Electron micrograph of an etched specimen of *Corvaspis* demonstrating the vaulted nature of the middle layer, the superficial dentine tubercles and the lamellar basal layer (BU 4473). (4,5) Etched SEM sections through the dermoskeleton of *Loricopteraspis dairydinglensis* showing (**4**), the basal lamellar bone layer and vertical strut belonging to the overlying cancellar (middle) bone layer and (**5**), osteons within the middle layer showing the radial arrangement of fibres about the osteons (BRSUG 27189). Relative scale bar: (1) 200 μm; (2) 500 μm; (3) 500 μm; (4) 52 μm and (5) 87 μm.

Tarlo, '64; Ørvig, '65; Moss, '68; Maisey, '88; Smith and Hall, '90; Smith, '91). However, these debates have been largely non-phylogenetic, relying instead upon stratigraphic or embryological order of appearance, or upon assumed evolutionary ''trends''. For instance, on the basis that cellular precedes acellular bone in the development and evolution of teleosts (and, erroneously, within osteostracans; Donoghue pers. obs.), Ørvig ('65) concluded that this trend must reflect the overall pattern of dermal bone evolution.

In phylogenetic terms, bone is first manifest as an acellular, matrix-rich mineralized tissue known as ''aspidin'' in pteraspidomorphs (Gross, '35; Denison, '67; Sansom et al., 2005a) (Fig. 2(1–5)) (bone appears to be absent from the oral skeleton of conodonts (Donoghue, '98) contra Sansom et al. ('92)). In pteraspidomorphs, aspidin is present both in the form of bone of attachment associated with the superficial dentine–enameloid tubercles (Fig. 2(1–3)), and comprises the whole of the underlying middle ''spongy'' (Fig. 2(1–3, 5)) and basal ''lamellar'' (Fig. 2(3,4)) layers of the dermoskeleton. However, the unifying characteristics of aspidin are few: it is acellular and is dominated by a rich organic matrix presumably consisting of collagen fibres (Donoghue and Sansom, 2002). Perhaps because of this paucity of structure, it has been compared to the acellular bone of attachment (cementum) met with in the teeth of higher vertebrates (Halstead Tarlo, '63). However, while this may be an adequate comparison for bone associated with the attachment of the superficial tubercles, the tissue comprising the bulk of the skeleton may find better comparison elsewhere. In particular, the basal lamellar layer, composed of a number of layers (Fig. 2(3,4), or ''ply'', between which the fabric of the fibre matrix varies in orientation, finds closer comparison to isopedin, a tissue that constitutes the basal layer of scales in extant osteichthyans (Wang et al., 2005). This distinction between basal and superficial bone accords with the observation that the superficial tubercles and deeper dermal bone layers appear to have been independently patterned (Westoll, '67; Donoghue and Sansom, 2002) and the expectation that they are derived from distinct cell lineages (Palmer and Lumsden, '87; Osborn and Price, '88). The tissue comprising the ''middle'' layer of pteraspidomorphs appears to be patterned in association with the superficial lamellar layer. In *Astraspis* and *Eriptychius*, this middle layer exhibits a spongy architecture, permeated by vascular canals (Denison, '67; Sansom et al., '97), while in most heterostracans this layer is organized into osteons about which the matrix fibres are radially arranged (Donoghue and Sansom, 2002) (Fig. 2(5)). Although some authors have used this difference in organization as the basis for a distinction between tissue types (Denison, '67; Halstead, '87; Smith and Hall, '90), the presence of a similarly spongy middle layer in the dermoskeleton of both plesiomorphic (e.g., corvaspid and tesseraspid) and derived (psammosteid) heterostracans suggests that the distinction between these two tissue grades is far from fundamental.

The bony tissues constituting the dermal scales of anaspids are poorly characterized, although they have been compared to aspidin (Gross, '38, '58; Janvier, '96a; Blom et al., 2002). The bulk of their dermal scales does appear to have been based on a fibre-rich organic matrix (Fig. 3(1,2)), but the fabric of this matrix is quite distinct from what is seen in aspidin and the fibres themselves appear to have been extrinsic rather than intrinsic (Donoghue and Sansom, 2002).

The thelodont dermoskeleton is composed solely of superficial tubercles (Fig. 4(5)), while in galeaspids there is only the equivalent of the lamellar basal layer of pteraspidomorphs (Fig. 3(3)). Thus, thelodonts possess only a bone of attachment that is comparable to aspidin. Meanwhile, the galeaspid dermoskeleton is exclusively composed of acellular bone and, although its surface often exhibits a tubercular ornament, it is devoid of dental tissues (Wang et al., 2005). Instead, the dermal armour of galeaspids is composed of a variant of isopedin. In addition to the two, alternating, circumferential collagen fibre fabrics that define the layers within the skeleton, there is a third, radial fabric (Fig. 3(3)). Finally, the tips of the tubercular ornament are composed of a spheritic bone that intergrades imperceptibly with the underlying laminar bone (Wang et al., 2005).

Osteostracans exhibit the first evidence of cellular bone in vertebrate phylogeny, manifest simultaneously in both the dermoskeleton and neurocranium (Stensiö, '27; Denison, '47) (Fig. 4(1)). Whether the basal isopedin layer of the dermoskeleton is cellular or acellular is a matter of interpretation (Gross, '56; Wang et al., 2005). The dermoskeleton of placoderms is composed of cellular bone (Fig. 3(4)) and, although both heterostracans and osteostracans show some evidence of bone resorption earlier in vertebrate phylogeny (Gross, '35; Denison, '52; Halstead Tarlo, '64), placoderms are the earliest group to

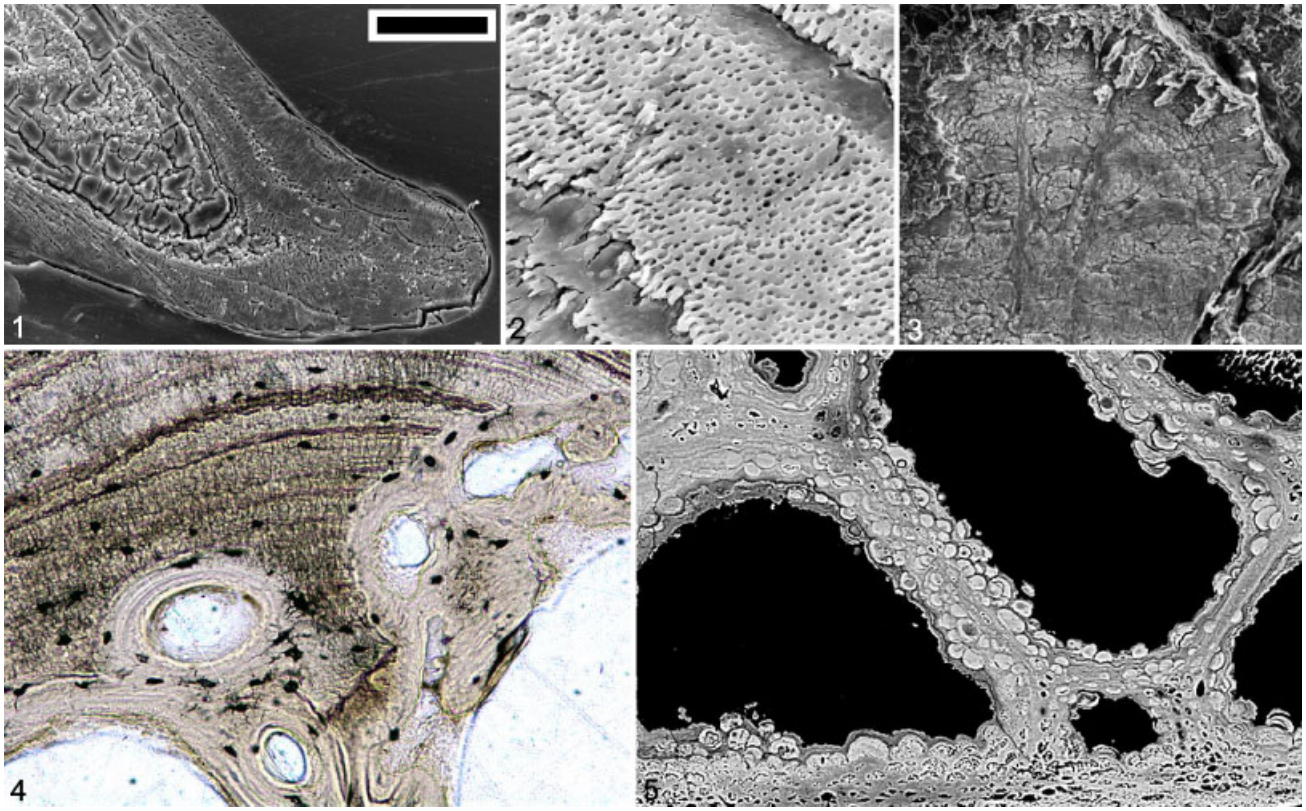

Fig. 3. Structure of the dermal bone in anaspids, galeaspids and placoderms. (**1**) Etched section through attachment point of a dermal scale of the anaspid *Birkenia robusta* showing the radial attachment fibres (BRISUG 27768). (**2**) Etched section of a dermal scale of the anaspid *Birkenia robusta* showing the tubules left behind by extrinsic matrix fibres (BRISUG 27769). (**3**) Eteched section through the superficial layer of the dermoskeleton in a polybranchiaspid galeaspid showing the three orthogonal sets of matrix fibres orientations (IVPP V12599.5). (**4**) Optical micrograph of the right postmarginal element of the *Bothriolepis canadensis* external skeleton demonstrating skeletal remodelling in the form of resorption spaces and secondary osteon development (MHNM 02-616). Scale bar 50 μm. (**5**) Backscatter electron micrograph of the left mixilateral element of the *Bothriolepis canadensis* external skeleton demonstrating spheritic mineralization of bone tissue in the dermal skeleton (MHNM 02-616). Scale bar 50 μm. Relative scale bar: (1) 40 μm; (2) 16 μm; (3) 19 μm; (4) 115 μm and (5) 63 μm.

show evidence of systematic remodelling of the skeleton (Fig. 3(4)). In association with this, some placoderms exhibit a peculiar form of spheritic bone (Ørvig, '68) that, were it not for its topology within the dermoskeleton, might readily (but erroneously) be interpreted as globular calcified cartilage (compare Figs. 3,53(5) and 5(1–5)).

Cellular bone remains an enduring characteristic of crown gnathostomes although, as aforementioned, acellular bone re-emerges in a variety of teleosts and amniotes (Meunier, '87).

Thus, it appears that cellular bone evolved from an acellular bone, and acellularity has arisen secondarily in a number of instances, often through distinct developmental pathways. While most forms of bone appear to be based on a collagenous matrix, and in some instances only the organic matrix remains (Meunier and Huysseune, '92), in galeaspids and placoderms, at least, there is evidence that bone can develop through spheritic mineralization in the absence of a collagenous matrix.

DENTINE EVOLUTION

Ørvig ('67) proposed a simple model of dentine evolution that centres on two characters: the inclusion or exclusion of odontoblasts within the mineralized matrix, and the polarity of the cell processes themselves. In this view, the most primitive state is represented by mesodentine, a cellular tissue with unpolarized cell processes that exhibit a reticulate branching pattern reminiscent of cellular bone. Indeed, mesodentine has been the source of the long-standing hypothesis that bone and dentine are closely related tissues that are derived one from another or from a common precursor, possibly represented by mesodentine

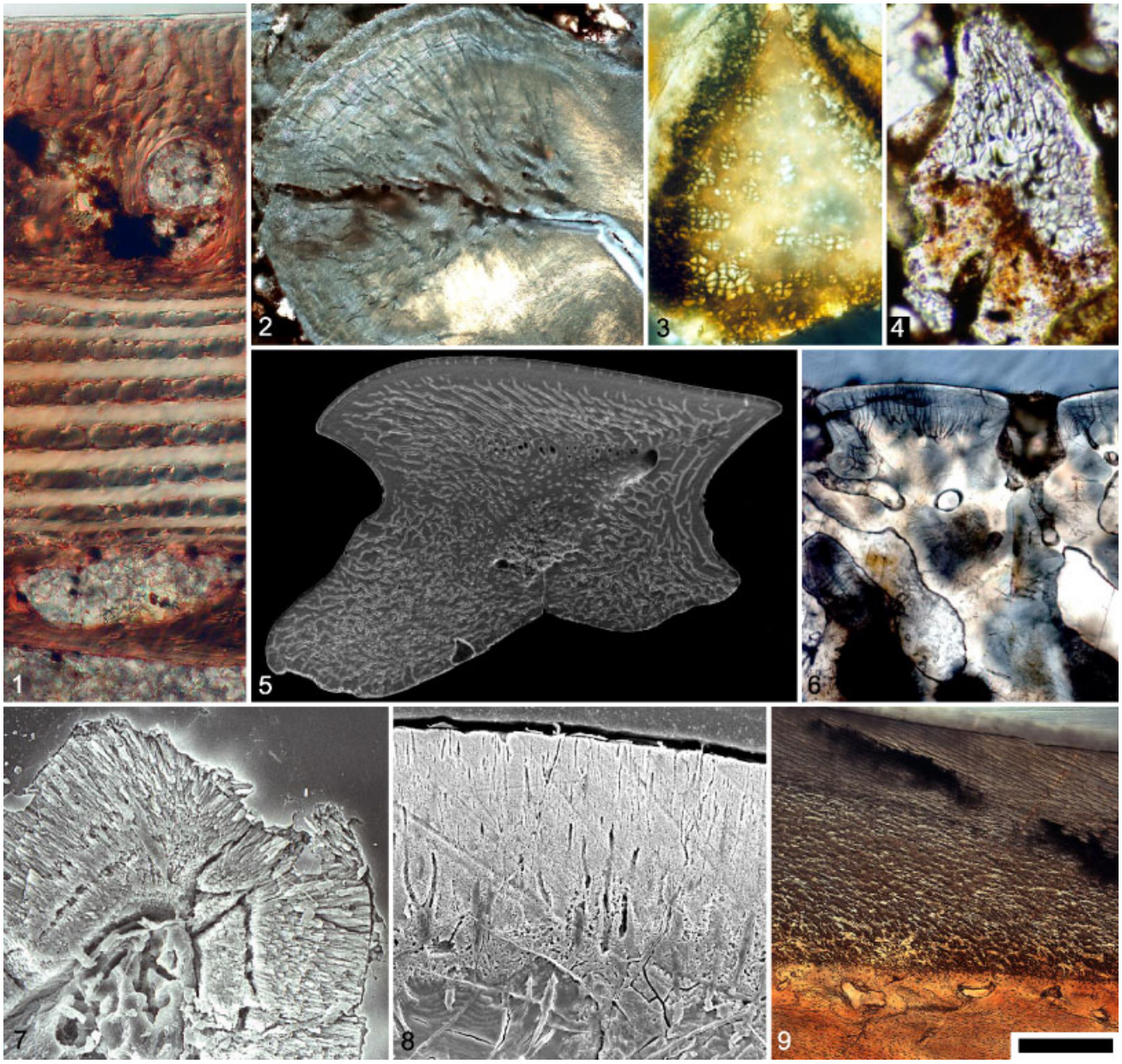

Fig. 4. (**1**) Nomarski interference optical micrograph of a section through the dermoskeleton and underlying endoskeleton of the osteostracan *Trematapsis* revealing a superficial layer of mesodentine, and a middle layer of isopedin (FM4109). (**2**) Nomarski interference optical micrograph of the coarse calibre dentine in *Eriptychius* (BU 4476) (**3**) Basal body of the conodont *Drepanodus* in cross-polarized light showing the extinction crosses in the radially arranged crystallites comprising spherules (BU 2694). (**4**) Nomarski interference optical micrograph of cellular mesodentine and cellular bone in the enigmatic Ordovician taxon *Skiichthys* (BU 2175) (**5**) Electron micrograph of a section through a dermoskeletal scale of *Thelodus* demonstrating a core of orthodentine and a thin superficial layer of enameloid (BRISUG 27770). (**6**) Nomarski interference optical micrograph of branching ''orthodentine'' in *Corvaspis*. (BU 4473). (**7**) Electron micrograph of an etched specimen of the conodont *Pseudooneotodus* illustrating perpendicular crystallite enamel surrounding the basal body (BU 2278). (**8**) Enameloid–dentine junction in the dermoskeleton of the heterostracan *Tesseraspis tesselata*, permeated by dentine tubules (BRISUG 27184). (**9**) Nomarski interference optical micrograph of junction between the dentine and parallel-bundled enameloid in the selachian *Odontaspis lamna* (BU 4474). Relative scale bar: (1) 55 μm; (2) 150 μm; (3) 170 μm; (4) 250 μm; (5) 173 μm; (6) 100 μm; (7) 10 μm; (8) 41 μm and (9) 125 μm.

itself (Halstead Tarlo, '64; Ørvig, '67; Halstead, '74, '87; Smith and Hall, '90). The next grade in this evolutionary transformation series is represented by semidentine, in which odontoblasts appear to have remained entrained within the mineralized matrix, but the cell processes are

strongly polarized in a single direction and show less of a tendency towards reticulation. Orthodentine, the most advanced grade, is the more typical condition for dentine. The cell processes are usually parallel sided and although they may show evidence of lateral branching, they remain distinct along their length.

Despite its endurance, Ørvig's model fails to accord with either the stratigraphic order of appearance or the phylogenetic branching order of the taxa in which these structural grades are encountered (Donoghue et al., 2000). Neither does it encompass the full range of dentine structural grades met with in early vertebrate phylogeny which includes a range of atubular histologies in addition to those characterized by Ørvig (Smith and Sansom, 2000).

The stratigraphically earliest and phylogenetically most primitive dentines known are met with in the conodonts, where dentine constitutes the so-called basal body. Conodonts themselves exhibit a wide range of structural grades including, in rare instances, tubular dentines characteristic of both Ørvig's orthodentine and mesodentine grades (Sansom et al., '94; Smith et al., '96a; Dong et al., 2005). More commonly, conodont dentine is represented by atubular lamellar and/or spheritic patterns of mineralization (Sansom, '96; Donoghue, '98; Dong et al., 2005) (Fig. 4(3)); these are also encountered among more derived vertebrates including primitive members of Chondrichthyes (Karatajuté-Talimaa et al., '90; Sansom et al., 2000).

Moving through phylogeny, pteraspidomorphs exhibit exclusively tubular dentines, and although these fit broadly within Ørvig's orthodentine grade, aside from the absence of entrained odontoblast cell spaces, pteraspidomorph dentines exhibit considerable variation, both in terms of the scale and branching architecture of the cell processes themselves (Fig. 4(2,6,8)). Orthodentine is also characteristic of thelodonts (Fig. 4(5)), although in some instances, the inter-process branching is so poorly organized that it is sometimes reminiscent of an acellular mesodentine (Gross, '68; Turner, '91).

Mesodentine is most widespread in the osteostracans. Here, cell processes exhibit the reticulate pattern of intertubule branching typical of mesodentine, although there is often a gradation to more orthodentine-like organization toward the outer surface of the dentine (Fig. 4(1)). Thus, the distinction between these two structural grades may not be fundamental, with mesodentine appearing to be a mere artefact of odontoblast retreat to the infilling of numerous, closely set pulp cavities.

Placoderms, the sister group of the gnathostome crown, are the only vertebrates to clearly exhibit semidentine, although orthodentine is also known from some placoderms (Smith and Johanson, 2003). Exceptions notwithstanding (Shellis, '83; Karatajuté-Talimaa et al., '90; Karatajuté-Talimaa and Novitskaya, '92; Appleton, '94; Sansom et al., 2000, 2005b), crown gnathostomes generally exhibit only orthodentine (Fig. 4(9)).

In summary, there is no phylogenetic evidence to support the trends proposed within Ørvig's model of dentine evolution. All grades of dentine are manifest among the earliest skeletonizing vertebrates. The earliest dentines are both atubular and tubular, and, structurally, the main grade of tubular dentine has always been on an orthodentine template, with mesodentine and semidentine autapomorphic to specific lineages.

ENAMEL/OID EVOLUTION

There has been considerable debate in the literature concerning the plesiomorphy of enamel, whether it is a tissue exclusive to tetrapods, or whether it is homologous with structurally/developmentally similar tissues in fish. The latter case has prompted the suggestions that enamel is a vertebrate symplesiomorphy (Maisey, '86) or even a vertebrate synapomorphy (Smith, '95).

Many of these debates have been non-phylogenetic and limited in scope to issues concerning the presence or absence of matrix proteins, structures and mineralization patterns (Shellis and Miles, '74; Moss, '77; Bendix-Almgreen, '83; Sire, '94; Sasagawa, 2002). A phylogenetic reading indicates that enamel and enameloid are merely grades of hypermineralized tissue that appear to have evolved independently in a number of instances (Donoghue, 2001).

Hypermineralized tissues that can be compared to enamel and enameloid are first encountered phylogenetically among conodonts (Fig. 4(7)) where the majority of the element ''crown'' is composed of a tissue that is structurally, topologically and apparently developmentally indistinguishable from enamel (Sansom et al., '92; Sansom, '96; Donoghue, '98, 2001).

In pteraspidomorphs, the hypermineralized tissue capping dentine tubercles of the dermoskeleton is enameloid (Fig. 4(6,8)). In *Astraspis*, it appears to be monocrystalline (Fig. 2(1)), while in arandaspids and heterostracans there is often a

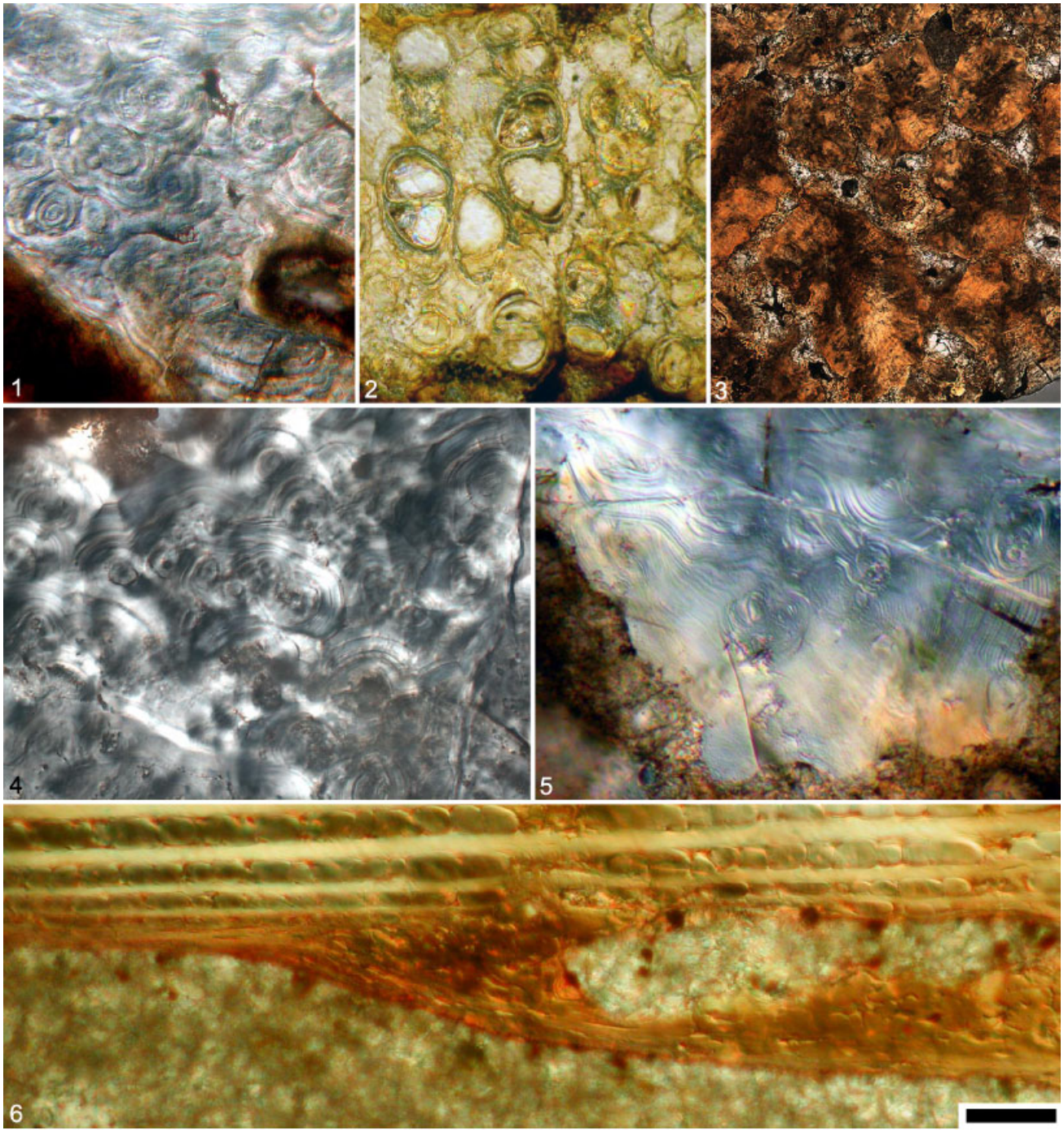

Fig. 5. Endoskeleton. Cartilages and perichondral bone. (**1**) Nomarski interference optical micrograph of globular calcified cartilage in the primitive chondrichthyan *Sinacanthus* (IVPP.V14325). (**2**) Nomarski interference optical micrograph of globular calcified cartilage in *Euphanerops*, an anaspid-like form from the late Devonian of Miguasha, Canada (MHNM 01-135A). (**3**) Nomarski interference optical micrograph of prismatic cartilage in the chondrichthyan *Akmonistion* (GN 1047.a1). (**4**) Nomarski interference optical micrograph of globular calcified cartilage from the Harding Sandstone and attributed to *Eriptychius* (BU 4475). (**5**) Nomarski interference optical micrograph of globular calcified cartilage in the neurocranium of a galeaspid (IVPP V12607.1). (**6**) Nomarski interference optical micrograph of cellular perichondral bone attached to the base of the dermoskeleton in *Trematapsis* (FM4109). Relative scale bar: (1) 50 μm; (2) 23 μm; (3) 75 μm; (4) 50 μm; (5) 53 μm; (6) 45 μm.

fabric of aligned crystallites (Fig. 4(8)), indicative of mineralization coordinated by matrix vesicles or collagen fibres (Sasagawa, '89, '97). Thelodonts exhibit only a thin superficial layer of monocrystalline enameloid (Fig. 4(5)), while enamel and enameloid are absent from galeaspids, osteostracans and placoderms (reports of enameloid in trematapsid osteostracans (Janvier, '96a) are not borne out by SEM study). Fibrous and single crystallite enameloids are encountered in the teeth and scales of both lineages of basal crown gnathostomes, the chondrichthyans (Fig. 4(9)) and osteichthyans. The enameloid covering the dermal scales of actinoptergygians (ganoine) exhibits similarities to enamel in its mode of development (Sire et al., '87; Sire, '94) even though its mineralized matrix is more closely comparable to enameloid. Although some (e.g., Moss, '77) have argued for homology between chondrichthyan and osteichthyan enameloids, differences in their modes of mineralization have led others to conclude that enameloid has evolved independently in each of these two lineages (Bendix-Almgreen, '83; Sasagawa, 2002). Meanwhile, ''true'' enamel is encountered along with enameloid in the teeth of actinopterygians (Smith, '92), and alone as the covering of teeth and scales in sarcopterygians (Smith, '89, '92).

Evidently, there is no clear pattern to the phylogenetic distribution of enamel and enameloid among early vertebrates. Although enamel is manifest first in conodonts, single crystallite enameloid predominates in the dermoskeleton of jawless vertebrates. Fibrous enameloids are present in basal crown gnathostomes, while ''true'' enamel appears to be an osteichthyan character. Generally, this reflects a pattern of increasing tissue complexity.

EVOLUTION OF CARTILAGE AND BONE WITHIN THE ENDOSKELETON

The vertebrate endoskeleton appears to have had an unmineralized origin. Its most plesiomorphic manifestation is actually in the acraniate chordate *Branchiostoma*, where the cartilaginous elements supporting the pharyngeal clefts (and buccal cirri) are perhaps homologous to components of the viscerocranium of hagfishes and lampreys (De Beer, '37), although attempts to derive homology even between the cranial cartilages of hagfishes and lampreys, have proven futile (Holmgren and Stensiö, '36). Hagfishes possess a more extensively developed viscerocranium, with additional cartilaginous supports for the fins and head structures such as the tentacles. In addition to these, lampreys also possess cartilaginous arcual elements that have been considered homologous to the vertebrae of gnathostomes (Janvier, '96a,b). However, at least at the structural and biochemical level, most of the cartilage is quite unlike that of more derived vertebrates. Each lineage possesses a unique suite of structural proteins (Wright et al., 2001). Nevertheless, it is likely that cartilages of this kind were present in the viscerocranium of all stem-gnathostomes bar placoderms, given that no mineralized equivalent to the hagfish–lamprey endoskeleton is present. This is lent support by the apparent discovery of (possibly only fortuitously) mineralized cartilage in an anaspid (Janvier and Arsenault, 2002) that, at the level of light microscopy (Fig. 5(2)), is structurally comparable to the cartilage of lampreys (Langille and Hall, '93).

The first evidence of mineralization within the endoskeleton are scraps of globular calcified cartilage associated with the Ordovician pteraspidomorph *Eriptychius* (Denison, '67) (Fig. 5(4)), although precisely what component of the endoskeleton they represent remains unknown. Otherwise, galeaspids provide the first evidence of a mineralized endoskeleton (Fig. 5(5)). Instead of a mineralized viscerocranium, this is in the form of lamellar and spheritic calcified cartilage in the neurocranium (Wang et al., 2005). Rather than a discrete box-like entity, the galeaspid braincase is a large expanse of cartilage plastered directly onto the base of the dermoskeleton, on the roof of the oralobranchial chamber and encompassing not only the brain but also covering the branchial chamber dorsally. Of course, these could be considered galeaspid peculiarities were it not for the fact that the braincase of osteostracans is similarly expansive, but also encompassing the pericardial chamber and pectoral girdle (Janvier, '84). Furthermore, although placoderms possess a discrete pectoral girdle, the braincase remains extensive, attached to the ventral surface of the dermoskeleton (Goujet, 2001). Thus, it is likely that such a braincase was common to the crownward members of the gnathostome stem (Janvier, 2001; Wang et al., 2005). Nevertheless, the composition of the neurocranium exhibits significant evidence of change, from the spheritically calcified cartilage, indicating absence of an organic framework in galeaspids (Fig. 5(5)), to the cellular perichondral bone lining a spheritically calcified cartilaginous core in osteostracans (Fig. 5(6)), to

the replacement bone in gnathostomes (Stensiö, '27, '32; Denison, '47; Wängsjö, '52; Janvier, '85). Mineralization of the appendicular skeleton is first manifest in the osteostracans as discs of spherulitic cartilage within the paired fins (Janvier et al., 2004).

Placoderms provide the first reflection of endoskeletal development that is generally (though erroneously (Donoghue and Purnell, 2005)) taken to be representative of vertebrates. All fundamental components of the endoskeleton are mineralized in at least some placoderms, including a jointed viscerocranium and axial and appendicular skeletons that show evidence of perichondral bone (Donoghue and Sansom, 2002). There are tenuous records of endochondral bone in osteostracans (Janvier, '85), placoderms (Denison, '78) and acanthodians (Denison, '79). However, endochondral bone is perhaps most safely considered an osteichthyan character.

THE EVOLUTION OF TISSUE ASSOCIATIONS AND CELLULAR INTERACTIONS

Although we may begin to piece together the evolution of individual skeletal tissues, a number of significant questions remain to be answered. What is the record of their interactions? Have skeletal tissues always been expressed and combined into the same stereotypical suites that we understand from living vertebrates? Is there any evidence early in skeletal evolution of experimentation with tissue combinations not found in living representatives (Halstead, '87; Sansom et al., '94; Hall, 2005)?

Before we progress, it is worth discussing potential circularity in our use of tissue topologies as a criterion in their identification, and subsequently drawing inferences based upon the perhaps inevitable conservation of tissue topologies through vertebrate phylogeny. However, while we have emphasized significance of topology, only in one instance (spheritic bone in the dermoskeleton of *Bothriolepis*) have we used topology as the principle character on which a tissue interpretation is based, and the conclusions that we draw below do not depend upon this identification.

Proposed examples of novel tissue associations early in phylogeny have been based upon controversial interpretations of tissue types and have not withstood scrutiny. For instance, it has been proposed that the conodont basal body was variably composed of dentine or globular calcified cartilage (Sansom et al., '92, '94; Smith et al., '96a). However, the structures on which identification of cartilage was based are not incompatible with an interpretation as dentine (Donoghue, '98). The interpretation that forms of dentine preceded bone in comprising the bulk of the dermoskeleton is based upon the description of what are two distinct tissues, one a variety of aspidin and the other a fine tubular dentine, as a single tissue, termed ''astraspidin'' by Halstead ('69, '87). However, despite variation in the structural appearances of bone, dentine, enamel/oid and cartilage among early vertebrates, from their first inceptions, the broad tissue associations and tissue topologies, appear to have remained consistent.

Conodonts, the earliest vertebrates with a mineralized skeleton, exhibit a condition that matches expectations for odontode-based development: enamel caps a dentine core and the tissues appear to have developed through appositional growth (Smith et al., '96a; Donoghue, '98). The odontode is the primary patterning unit of the dermoskeleton and is responsible for the development of both teeth and the dermal denticles (Ørvig, '68, '77; Reif, '82; Donoghue, 2002). In developmental models, the developing odontode unit is known to give rise to two distinct cell populations—the odontogenic component, which is responsible for the teeth and dermal tubercles of enamel/oid, dentine and bone of attachment; and the skeletogenic component, which is responsible for the bone of the jaw and probably the scale bases and plates of fishes (Palmer and Lumsden, '87; Osborn and Price, '88; Smith and Hall, '90; Sire and Huysseune, 2003). The independent patterning of these two cell lineage derivatives is apparent from the first manifestations of the dermoskeleton, in instances where both of these components are present (Westoll, '67), and in instances where the derivatives of only the odontogenic (thelodonts, chondrichthyans) or skeletogenic (galeaspids) population are present (Donoghue and Sansom, 2002). Although much has been made of the modifiability of the odontode (Schaeffer, '77; Reif, '82; Smith and Hall, '90, '93), there is little real evidence of its variation beyond the presence or absence of enamel/oid which, given that its induction occurs at the terminal end of an epigenetic cascade (Lumsden, '87), must in mechanistic terms amount to a minor case of heterochrony.

The developmental and phylogenetic dichotomy between the dermal and endoskeletons has been established at least since Patterson ('77). There is

no evidence of cartilage or perichondral bone in the dermoskeleton and instances where dermal bone and cartilage do occur in close association does not belie this distinction (Patterson, '77; Hall, 2005). Interestingly, however, the evolution of pharyngeal denticles and teeth suggests that there may be some evidence of an evolutionary link between the dermal and oral skeleton. Both teeth and dermal tubercles have long been interpreted as derivatives of the dermoskeleton and, indeed, it has traditionally been proposed that teeth evolved from specialized dermal scales positioned within the mouth or pharynx (Nelson, '69). However, experimental analysis of tooth development has revealed that teeth can only develop through interaction of endoderm and neural crest-derived ectomesenchyme and, thus, there is a fundamental embryological distinction between teeth and dermal scales (Smith and Coates, '98, 2000, 2001). The logical extension of this is that teeth are part of the endoskeleton (viscerocranium) and here the dermal–endoskeletal distinction breaks down (Donoghue and Sansom, 2002). Even the proponents of the ''teeth are not scales'' hypothesis argue that they have a common (albeit obscure) evolutionary origin. In other words, the odontode first arose within one or other skeletal system; a phylogenetic reading indicates plesiomorphy of the odontode within the viscerocranium. An attempt has been made to unite teeth and scales, along with all neural crest skeletal derivatives into a skeletal system distinct from the dermal and endoskeletons (Hall, '98, 2000). However, this serves little more than to emphasize the manner in which neural crest derivatives transcend traditional germ layer distinctions, and records the expansion of neural crest potentiality, but not the evolution of the skeletal systems or modules themselves.

EVOLUTION OF SKELETAL TISSUES: A SUDDEN OR GRADUAL EMERGENCE?

Despite the diversity of tissue types encountered among early skeletonizing vertebrates, from their origin, they have been arranged according to the embryological divisions and modules that we understand from model laboratory animals such as the mouse. From this, we can conclude that there are no fundamental distinctions between the various grades of bone, dentine, enamel/oid and cartilage. Given this, concerns that unfamiliar early skeletal tissue types have been shoe-horned into inappropriate concepts of modern tissues (Halstead, '87), appear unfounded. The question of why there is such a high diversity of tissue structural grades among early skeletonizing vertebrates, however, remains.

One part of the explanation lies with differing patterns of mineralization. Tor Ørvig identified two main types: inotropic, in which mineralization is organized by organic matrix, and spheritic, in which crystal nucleation is independent, occurring in the absence of such a matrix (Ørvig, '51, '67). Spheritic mineralization, widespread among invertebrates (Ubukata, '94), generally occurs only under teratological conditions in the skeletal tissues of living vertebrates (Shellis, '83; Appleton, '94; Kierdorf et al., 2000). Considering the fact that spheritic mineralization of dentine, cartilage and bone appears to have been widespread among early skeletonizing vertebrates, the protein-based architectural constraints on mineralization may not have been well established early in vertebrate phylogeny.

A more complete explanation has begun to emerge from the discovery that the diversity of structural proteins involved in vertebrate skeletogenesis arose episodically through vertebrate phylogeny. Kawasaki and Weiss (2003) and Kawasaki et al. (2004) studied the phylogenetic relationships and distributions of structural protein-coding genes implicated in vertebrate skeletogenesis. They argue that many such genes constitute a secretory calcium-binding phosphoprotein (SCPP) family, all derived through one or more gene duplication events from an osteonectin-like ancestral gene. Their hypothesis that the diversity of SCPPs correlates with tissue complexity accords with the data from early skeletonizing vertebrates. Although there is a diversity of structural grades, these are invariably of very low complexity. Recall, for instance, the plesiomorphy of atubular dentines and unmineralized cartilages, and the spheritic modes of mineralization exhibited by early dentines and cartilages (Ørvig, '67). Given that the diversity of SCPPs does not increase until after the origin of osteichthyans, Kawasaki proposes that the causal gene duplication event did not occur until after the divergence of the chondrichthyan and osteichthyan stem lineages (Kawasaki et al., 2004).

Although the histological data accord broadly with the hypothesis of Kawasaki et al. (2004), their inference of the precise timing for the duplication event does not find a good match with the entrenchment of tissue structural complexity that

was certainly achieved before the divergence of placoderms, and possibly earlier, in ancestors shared with osteostracans, a group in which the majority of characteristic vertebrate skeletal tissues are manifest. However, the inferred phylogenetic position of the gene duplication event remains open to question because the study by Kawasaki et al. (2004) crucially lacks data from chondrichthyans and, thus, on the basis of the available data, it is not possible to discriminate at what stage in phylogeny, between the rooting of lampreys and osteichthyans, the duplication of the osteonectin-like ancestral gene occurred. Nevertheless, Kawasaki et al. (2004) specifically tried to constrain this by attempting to date the absolute timing of SCPP gene divergence using molecular clock theory and comparing this date to the timing of divergence of extant vertebrate lineages established in an independent molecular clock study by Kumar and Hedges ('98). Thus, Kawasaki et al. (2004) arrived at their conclusion of an event postdating the split of chondricthyans and osteichthyans. However, the divergence time estimates derived from molecular clock studies are far too imprecise for the sort of inter-analysis correlation attempted by Kawasaki et al. (2004). The errors on Kumar and Hedges' ('98) estimate for the actinopterygian–sarcopterygian split is 450 Ma $\pm$ 35.5 Myr, which overlaps with the 528 Ma $\pm$ 56.4 Myr estimate for the chondrichthyan–osteichthyan split. In fact, the errors, as presented, are a very conservative estimation of the real errors inherent in their calculation (Donoghue et al., 2003). More worryingly, the point estimates (not including errors) for bird–mammal split in both analyses are some 37 Myr astray, even though both analyses were ultimately calibrated using fossil data for this divergence event–directly in the case of Kumar and Hedges ('98), and indirectly, via Wang et al. ('99), in the case of Kawasaki et al. (2004).

One further point is worth considering. Although duplication of individual genes occurred relatively frequently during vertebrate phylogeny, the vast majority of gene duplication appears to have occurred during focussed episodes or, as is appearing increasingly likely, during whole genome duplication events (Furlong and Holland, 2002b, 2004; Dehal and Boore, 2005; Panopoulou and Poustka, 2005). We should, of course, remain cautious and avoid interpreting data in the light of this largely untested hypothesis. Nevertheless, given this context and the absence of constraining data from chondrichthyans, it might be likely that the origin of SCPP diversity is the product of gene duplication associated with the origin of gnathostomes.

Even if it were to be accepted that the origin of SCPP diversity is a gnathostome phenomenon, this does not provide much further constraint over the timing of duplication with respect to tissue complexity (Donoghue and Purnell, 2005). This is because the duplication event could have occurred at any point within the extensive gnathostome stem-lineage intermediate of lampreys and crown gnathostomes, before the origin of conodonts, after the origin of placoderms, or at any point in between, and duplication, in itself, only provides a potential for subsequent histological expression. Thus the divergence of SCPP genes might have preceded the origin of vertebrate tissue complexity.

Such questions aside, Kawasaki et al. have made an important discovery in the origin of SCPP diversity, one that provides an explanation for the diversity within vertebrate tissue types and the diminishment of this diversity through early vertebrate phylogeny. This could be considered developmental plasticity but is more appropriately considered an artefact of the absence of the structural proteins involved in skeletogenesis in more derived, living vertebrates.

CONCLUSIONS

Many of the common perceptions of skeletal tissue evolution are based on stratigraphic and embryological order of appearance or on morphoclines, the selection of an obvious morphological character and the ordering of its variation into an intuitive pattern (e.g., Ørvig's ('67) model of dentine evolution). Demonstrating the problems inherent in these styles of identifying evolutionary trends, long-standing perceptions quickly break down when viewed in a cladistic context and, further, they are not necessarily replaceable by other simple, definable trends.

Despite demonstrable variation within the major vertebrate skeletal tissues (i.e., bone, dentine, enamel and cartilage) of basal vertebrates, the suggestion (Halstead, '87) that this variation has been forced into an unnatural classification appears to be unfounded. The pattern of variation may be better explained by episodic duplication events in SCPPs through vertebrate phylogeny and the resulting increases in structural complexity within these tissue types (Kawasaki and Weiss, 2003; Kawasaki et al., 2004). The unusually high degree of diversity among basal vertebrates may

simply be an artefact of the predicted relatively low diversity of SCPPs and a corresponding lack of constraints on the mineralization of their skeletal tissues.

Whilst the variation within tissue types does not demand subdivision of these categories, challenges to the disparity *between* the commonly recognized skeletal tissue types appear to be unfounded. The topology, associations and histology of these tissue types are consistent with the hypothesis that these tissue types are fundamentally distinct from their origin (Moss, '64; Halstead, '87; Maisey, '88).

Finally, despite the fact that neural crest contributions extend between the skeletal systems, the phylogenetic distinction between the dermoskeleton and endoskeleton remains unchallenged since its original proposal (Patterson, '77).

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