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Cartilage is a metazoan tissue; integrating data from nonvertebrate sources

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Abstract

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Connective tissues are responsible for much of the variation in morphology that we see today. Cartilage is a type of connective tissue that is often considered to be restricted to vertebrates, however, cartilaginous tissues are also found within invertebrates. Unfortunately, most definitions and classification schemes for cartilages suffer from a strong vertebrate bias, severely hampering the efforts of those who have attempted to include invertebrate tissues as cartilage. To encompass all types of cartilage, current classification systems need to be expanded. Here we present vesicular cell-rich as a new cartilage classification. Invertebrate cartilages, comparable to vertebrate cartilages at both cell and tissue levels, are composed of similar molecules, yet the extent to which they may be homologous is unknown. One option for studying the evolution of tissues is to adopt molecular phylogenetic approaches. However, the paucity of published molecular data makes addressing the evolution of cartilage using molecular phylogenetic approaches unrealistic at this time. Cartilage likely evolved from a chondroid connective tissue precursor, and may have been independently derived many times. The appearance of cartilaginous tissues of unknown phylogenetic affinities in such a wide diversity of animal groups warrants further investigation.

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Introduction

Connective tissue

Connective tissues are most appropriately considered as a spectrum of tissue types that differ in the organizational complexity of their extracellular matrices, providing the characteristic features for distinguishing types of connective tissue. At the most basic level, extracellular matrices are composed of two types of molecules, ground substance (glycosaminoglycans, proteoglycans), and fibrous proteins (largely but not exclusively collagen) (Junqueira *et al.* 1998). Much of the diversity in connective tissues arises from different relative amounts of these two components. The extracellular matrix of connective tissue is organized into three regions – the glycocalyx, which immediately surrounds the cell membrane; the pericellular matrix, which interacts with the glycocalyx; and the remaining extracellular matrix outside the pericellular matrix. All animal cells have a glycocalyx, and most have some degree of pericellular matrix. The structural organization of the remaining matrix components allows further characterization of connective tissue into more specific types (Maclean and Hall 1987).

Vertebrate connective tissue types can be distinguished in histological section by the relative amounts of the two different extracellular matrix components, and the orientation of fibrous proteins. For example, regular dense connective tissue (e.g. ligament or tendon) is identifiable by its parallel, compact fibres (Ham and Cormack 1979), distinguishable from irregular dense connective tissue by the directionality of the fibres, and from fibrocartilage by the cell morphology and pericellular matrix. However, along the spectrum of

connective tissue diversity there exists significant histological overlap between different tissue types, sometimes confounding tissue identification. One type of connective tissue deserving further consideration is cartilage.

Cartilage

Cartilage is not a skeletal support tissue found exclusively in vertebrates, some authors have been aware that cartilage is also found within nonvertebrate taxa (Person and Philpott 1969a; Hall 1978, 2004; Person 1983; Robson *et al*. 2000; Wright *et al*. 2001). Certain invertebrate cartilages are indistinguishable from vertebrate cartilage, but others demonstrate distinct histological morphologies. The presence of cartilage or cartilage-like tissues in many distantly related clades (Fig. 1), which include, in addition to Vertebrata, Brachiopoda (Reed and Cloney 1977; Stricker and Reed 1985), Mollusca (Tompsett 1939; Raven 1958; Crowden 1967; Person and Philpott 1969a), Annelida (Person and Mathews 1967; Person and Philpott 1969a), Arthropoda (Crowden 1967; Person and Philpott 1969a,b; Makioka 1988; Wright *et al*. 2001), Hemichordata (Kowalewsky 1867), and Cnidaria (Schaffer 1930; Person and Philpott 1969a), suggests that early in metazoan history connective tissue gained the ability to organize with cartilage-like properties (chondroid connective tissue). *Chondroid connective tissue* should not be confused with *chondroid bone*, an example of a tissue intermediate between vertebrate cartilage and bone (Beresford 1981). By extension, all metazoans should be able to form a connective tissue with cartilage-like properties under appropriate circumstances. Animals that do not have these tissues have found other solutions to the structural problems addressed by chondroid connective tissues, and may have lost the ability to form such tissue over time.

Not all cartilage-like connective tissues warrant the label cartilage. At what point should a connective tissue with cartilage-like extracellular matrix properties be identified as cartilage, and what does this mean for how we assess the homology of such tissues? The ability to identify cartilage and/or cartilage-like (chondroid) connective tissues is a prerequisite to answering these questions.

What is cartilage?

Considering the diversity of tissues that have been called cartilage and the complexity of these tissues, it comes as no surprise that working definitions and classifications of cartilage were developed in the context for which they were employed (Moss and Moss-Salentijn 1983). Vertebrate cartilage has been classified based upon positional (e.g. articular cartilage), developmental (e.g. primary vs. secondary cartilage), and histological (e.g. hyaline cartilage) criteria. Of these, those based upon histological analysis are by far the most useful, because other classifications rely heavily on taxon-specific characters. Taylor *et al*. (1994) demonstrate the utility of using histological analyses for classifying previously undescribed skeletal tissues in the yellow perch (*Perca flavescens*) through comparisons of the histology of perch tissue with other vertebrate skeletal tissues.

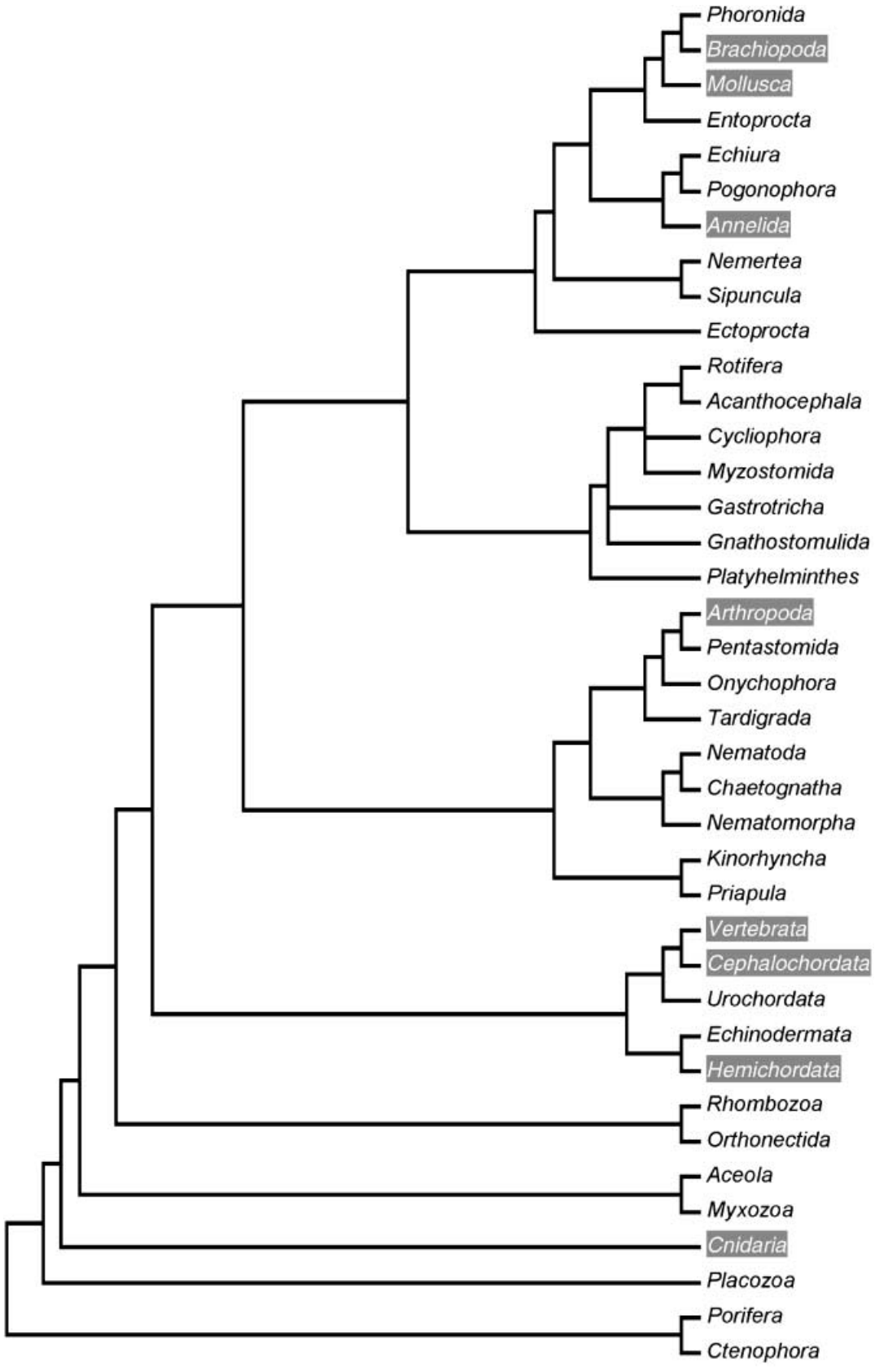
Histological classification

On the basis of histology, mammalian cartilages can be classified as hyaline, fibrous, or elastic. Images of mammalian hyaline cartilage are prevalent in histology textbooks as typifying cartilage at the histological level (Fig. 2a). Hyaline cartilage has an abundant metachromatic matrix, and the chondrocytes (cartilage cells) exhibit a rounded morphology. Hyaline cartilage differs from elastic cartilage in that the extracellular matrix of the latter contains elastin in addition to collagen (fibrous protein) and chondroitin sulphate (glycosoaminoglycan) (Ham and Cormack 1979). Fibrocartilage is a tissue in which the extracellular matrix has a higher fibrous content, similar to dense connective tissue but with cells that exhibit a typical rounded chondrocyte as opposed to the flattened morphology of a fibrocyte (Beresford 1981).

Detailed histological analysis of the cartilages found in teleost fish led Benjamin (1990) to establish yet another broad category of cartilage, *cell-rich* cartilages, for cartilages where > 50% of the tissue volume is comprised of cells rather than extracellular matrix. Cell-rich cartilages can be subdivided into a number of distinct classes, based upon histological features of the tissue. These include the hyaline-cell cartilages (having cells with chromophobic cytoplasm), *Zellknorpel* (having a more rigid matrix than hyaline-cell cartilages), and cell-rich cartilages (Schaffer 1930; Benjamin 1990) that can be further categorized into hyaline, fibro- and elastic cell-rich cartilages based upon matrix properties.

Classification of cartilaginous tissues into the aforementioned types should not be restricted to vertebrate tissues, given that many invertebrates have cartilage. Although consideration of tissue homology is important, the appearance of cartilage in two distinct groups says nothing of its qualities, only its origin. The primary concern here is the identification and categorization of connective tissues as cartilage, which should be based on histological properties

Fig. 1—Distribution of tissues previously described as cartilage (grey) within the metazoa. Phylogenetic relationships depicted are based upon analysis of published phylogenetic hypotheses derived from molecular sources (A. G. Cole unpublished data). Cartilage has been described within the lophophore and pedicle of brachiopods, associated with the radula in gastropod molluscs, and in numerous places within the cephalopod molluscs, supporting the feeding tentacles in sabellid polychaetes and gills within horseshoe crabs. Enteropneust hemichordates have an internal skeleton supporting the gills and proboscis, and tissues resembling cartilage have been reported within the mesoglea of some cnidarians. See text for full references.



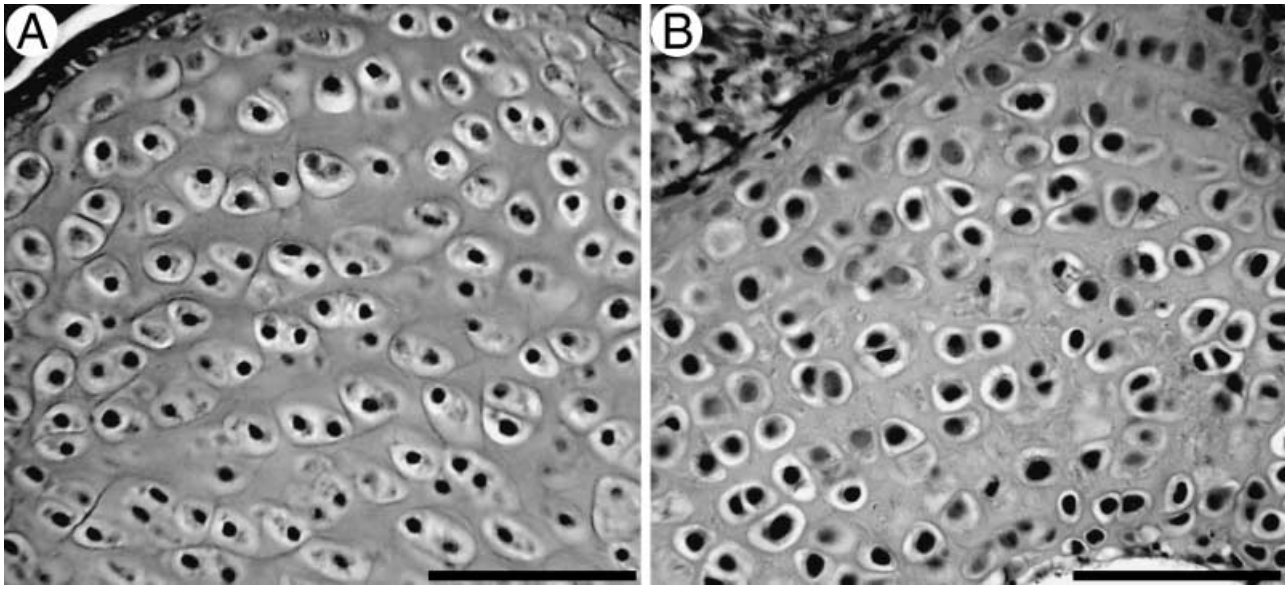


Fig. 2—Histology of cartilage within vertebrates and cephalopods from paraffin sections. —A. Articulating cartilage in the leg of a dwarf African frog (*Hymenochirus*). —B. Funnel cartilage in a squid (*Illex*). Masson's trichrome staining, scale bar = 0.5 μm.

regardless of phylogenetic origin. Restricting the definition of cartilage to include only tissues that can be clearly linked through common descent (homology) would disallow cephalopod cartilage the label 'cartilage', thereby eliminating the ability to identify cartilage based upon histological criteria.

Cephalopod cartilage is largely indistinguishable from mammalian hyaline cartilage at the light microscopic level (Fig. 2b). However, at the ultrastructural level these tissues differ; cephalopod chondrocytes have cell processes that make cell–cell connections with neighbouring chondrocytes (Bairati *et al*. 1998), similar to vertebrate osteocytes (Holtrop 1990), whereas there are no reports of such cell–cell connections in any vertebrate cartilages (Kuettner and Pauli 1983). As terminally differentiated cells, these two vertebrate cell types secrete different types and amounts of extracellular substances resulting in different extracellular matrices. Some invertebrate cartilages (e.g. those in sabellid polychaetes) can be distinguished from vertebrate cartilages by the presence of large vacuoles within the chondrocytes. This feature makes these invertebrate chondrocytes more similar in histological appearance to vertebrate notochordal or even small adipose cells (Schaffer 1930). Molluscan radular cartilages have been reported to contain myoglobin, the oxygen-binding protein, within the chondrocytes and cartilage matrix (Person 1983). Structural differences between chondrocytes in these invertebrate cartilaginous tissues are not sufficient to warrant abandoning the designation 'cartilage'. Rather, they suggest that the current classification schemes need broadening to encompass the large spectrum of cartilaginous tissues.

Broadening cartilage classification: vesicular cartilage To facilitate the inclusion of all invertebrate cartilages into current schemes of cartilage classification, we propose adding a new category to Benjamin's (1990) cell-rich cartilages: *vesicular cell-rich cartilage*. The distinguishing feature of this type of cartilage would be the presence of large vesicles or vacuoles within the chondrocytes that augment the physical properties of the matrix. Within this new category would fall the cartilages within the tentacles of the sabellid polychaetes (Schaffer 1930; Person and Mathews 1967), the branchial cartilages of the horseshoe crabs (Person and Philpott 1969a,b), the radular cartilages of molluscs (Raven 1958; Person and Philpott 1969a), and possibly the notochord of chordates (Olsson 1965; Schmitz 1998) to name just a few.

The chordate notochord is an interesting tissue worth further mention. It provides skeletal support, is cellular, and the large vesicular cells are surrounded by an extracellular sheath containing both polysaccharides (including chondroitin sulphate; Welsch *et al*. 1991) and collagen types I and II (Eikenberry *et al*. 1984). Despite these characteristics, most researchers working on vertebrates would not consider the notochord to be cartilaginous, but rather to be epithelial; the large vacuolated cells are connected to one another by desmosomes and gap junctions and no extracellular matrix separates the cells (Schmitz 1998). The extracellular matrix molecules produced by the notochordal cells are restricted to the acellular sheath surrounding the notochord itself, and to the large vacuoles within the cell bodies.

Expanding the classification of cartilage to include tissues of the cell-rich vesicular cartilage type could permit the inclusion of some vertebrate notochords as a type of cell-rich

cartilage, representing either a highly derived condition where the matrix separating the individual cells has been eliminated, or an ancestral condition indicating an epithelial origin for cartilage. Alternatively, the epithelial nature of the notochord could represent an example of a second, independent evolution of a cartilaginous tissue within the chordates. The fact that notochord sheaths contain type II collagen (see below) raises interesting issues that go beyond this paper, including whether type II collagen should be considered a notochord collagen first, and a cartilage-specific collagen secondarily (Robson *et al.* 2000).

Identifying cartilage

Chondrocytes

Vertebrate chondrocytes are large round cells, whereas fibrocytes and osteocytes are usually more flattened with elongated processes. However, among vertebrate skeletal tissues there is considerable overlap in cell morphology relative to identified tissue type (e.g. Taylor *et al.* 1994). Spherical cell morphology is critical for the production of cartilage-specific matrix molecules within vertebrates (see Benjamin *et al.* 1994 for review). Often, designation of a tissue as a type of cartilage ultimately depends upon the presence of chondrocytes within the tissue (e.g. Witten and Hall 2002). However, identification of a chondrocyte is based on what appear to be rather tautological principles. A chondrocyte is a cartilage cell, identifiable by the fact that it is found within a cartilaginous matrix. In culture, cells are designated as chondrocytes when they begin to secrete the matrix that ultimately forms the cartilage. Interestingly, even stromal cells (fibroblasts) from adipose tissue can be induced to produce cartilage matrix when cultured in conditions that support a three-dimensional cell morphology (Erickson *et al.* 2002). Vertebrate chondrocytes secrete type II and X collagens and chondroitin sulphates whereas osteocytes secrete type I collagen, bone sialoprotein, osteonectin, osteocalcin and osteopontin. Therefore a cartilage cell, or chondrocyte, is distinguishable from an osteocyte or fibroblast largely by the *combination* of molecules that it secretes into its extracellular matrix. Hall (1970a,b) even suggested that the composition of the extracellular matrix, in particular synthesis of chondroitin sulphates, regulates the fate of connective tissue stem cells as chondrocytes as opposed to becoming osteocytes.

Extracellular matrix

Although it has become apparent that some of the molecules that constitute the extracellular matrix of vertebrate cartilage are largely restricted to these tissues, there are very few of these molecules, and in some cases their cartilage specificity is suspect. Nonetheless, vertebrate cartilage has been, and continues to be, recognized by the synthesis of a handful of specific molecules. The most prominent member of this list is type II collagen, which is consistently used by tissue-culture researchers as an indicator of chondrocyte differentiation (e.g. Erickson *et al.* 2002), the rationale being that cells synthesize cell-specific molecules and that production of those molecules is diagnostic for the cell type, even if other characteristics are not evident.

Collagen One distinction between cartilage and other skeletal tissues is that in vertebrates cartilage has a high proportion of type II collagen, whereas bone and fibrous connective tissue utilizes type I collagen. Although type II collagen is abundant in the cartilage of vertebrates, it is not restricted to cartilage. Type II collagen is found in noncartilaginous tissues such as the vitreous humor of the eye (Ayad and Weiss 1984), the developing corneal epithelium (Hayashi *et al.* 1988), and epithelial basement membranes – during epithelial–mesenchyme interactions (Wood *et al.* 1991).

However, as type II collagen is much more abundant in tetrapod cartilage than in these other tissues, the practice of utilizing abundant type II collagen as a marker for cartilage differentiation remains useful for studies of tetrapod cartilaginous tissues. Abundant type II collagen is not a reliable marker outside of tetrapods, such as in many teleost fishes where type II collagen antibodies fail to recognize some cartilaginous tissues (Benjamin and Ralphs 1991). In addition, Benjamin and Ralphs (1991) report type II collagen antibody expression in the bone of some teleost fish and three species showed extensive staining for collagen II throughout the dense connective tissue. If type II collagen is regarded as exclusively a cartilage molecule, then its expression in bone or connective tissue is unexpected, indeed it is contraindicated. However, most vertebrate cells contain the gene for type II collagen, and this collagen is regularly expressed by osteoblasts in the early stages of bone formation, usually in association with initial deposition of osteoid, while it is rarely seen once the matrix mineralizes (Scott-Savage and Hall 1979; Jacenko and Tuan 1986). Although type II collagen is the defining feature of primary cartilage in terrestrial vertebrates, both secondary cartilage [cartilages arising on membrane bones relatively late in development (Beresford 1981)] and shark cartilage contain high proportions of type I collagen in addition to type II collagen (Rama and Chandrakasan 1984).

If we look at the basal-most extant craniate chordates, the agnathans (e.g. lamprey and hagfish), we find both collagen-based (in hagfish type 2) and noncollagen-based (in hagfish types 1 and 3, and all lamprey) cartilages (Wright *et al.* 1998, 2001; Robson *et al.* 2000). Despite the absence of type II collagen from the cartilages of agnathans, this molecule is expressed in the notochord, leading Robson *et al.* (2000) to suggest that this collagen type 'originated as a notochord protein, and only became the predominant structural protein of cartilage matrix some time after the divergence of the jawless fish from the vertebrate ancestral line' (p. 290).

Following this assertion, should cartilage-like tissues be found in other metazoan phyla, they would lack type II collagen. To date, type II collagen has not been found in any invertebrate. Whether or not type II collagen is also utilized in the notochord of other nonvertebrate chordates, the Urochordata, is not known. Investigations considering the evolution of fibrillar collagens have revealed that vertebrate collagen types are more closely related to one another than to their invertebrate counterparts, indicating that the diversity of collagen types found in vertebrates evolved within the vertebrate lineage (Boot-Handford and Tuckwell 2003). All the above points indicate type II collagen is not required to form cartilaginous tissues, and thus should not be used to define cartilage as a tissue, but remains useful for defining subtypes of cartilage such as vertebrate hyaline cartilage.

Invertebrate cartilage collagens Because collagen sequences are absent from arthropod genomes, Boot-Handford and Tuckwell (2003) claimed that arthropods lost the fibrillar collagens. Arthropods are known to have cartilage and cartilage often utilizes collagen as its fibrous protein, therefore it will be instructive to determine which fibrillar protein is utilized in horseshoe crab cartilage. To date, the cranial cartilage of cephalopod molluscs is the only nonchordate cartilage for which the collagen has been analysed (see Kimura and Karasawa 1985; Sivakumar and Chandrakasan 1998; Bairati *et al.* 1999; Sivakumar *et al.* 2003).

Kimura and Karasawa (1985) compared skin and cranial cartilage collagens from the squid *Todarodes*, and suggested that collagen from both sources is derived from the same gene product, with post-translational modification in proline hydroxylation in the cartilage forms. Analysis of the collagen extract revealed two α chains, termed α1 and α2, with the structure $(\alpha1)_2(\alpha2)$. Homology to vertebrate type I collagen was proposed based on this structure and amino acid content (Kimura and Karasawa 1985). Interestingly, the squid collagen has more glycosylated hydroxylysine than vertebrate type I collagen, and in this respect, is more similar to vertebrate type II collagen (Kimura and Karasawa 1985).

Bairati *et al.* (1999) investigated collagens in the cranial cartilage of the cuttlefish *Sepia officinalis* using immunohistochemistry and noted that antibodies against almost all vertebrate collagens gave moderate reactivity of the extracellular matrix. The two exceptions were mammalian type I antibodies, which showed no reactivity with cuttlefish cartilage, and rat type V antibodies, which showed intensive immunoreactivity of the entire cartilage extracellular matrix, comparable to that achieved with antibodies raised against cuttlefish cartilage antigen. The pepsin-soluble collagen extract used as antigen to produce cuttlefish antibodies gave a similar electrophoretic pattern to that observed by Kimura and Karasawa (1985) and hence was also considered similar to vertebrate type I collagen (Bairati *et al.* 1999). Cross-reactivity between *S. officinalis* cartilage and the various vertebrate collagen antibodies, in particular a type V collagen antibody, suggests that there is a cuttlefish collagen that is similar to vertebrate type V. Sivakumar and Chandrakasan (1998) and Sivakumar *et al.* (2003), purified collagen from *S. officinalis*, and reported it to be similar to vertebrate type V or XI based upon the three isolated subunits of molecular weights 105, 115 and 130 kDa. *Sepia officinalis* cartilage also contains another collagen which is unlike any of the characterized vertebrate collagens (Rigo and Bairati 2002).

The accumulation of biochemical work on the major collagens of the cranial cartilage in *Sepia* strongly suggests that its cartilage contains more than one type of collagen molecule, and that at least one of these is similar to the minor collagens found associated with vertebrate bone (type V) and cartilage (type XI). Collagen isolated from the sea pen *Veretillum cynomorium*, a basal cnidarian, is biochemically very similar to vertebrate type V collagen (Tillet *et al.* 1996). A predominance of type V collagen within the cartilaginous tissues of arthropods and annelids would provide strong support that type II collagen is a molecule novel to the chordate lineage, and was subsequently co-opted into cartilage as the major collagen. Predominant type V collagen would also support the assertion of Garrone (1998) that the minor collagens, such as type V, are ancestral (see also Exposito and Garrone 1990). Collectively, it seems the ancestral fibrillar collagen utilized in cartilage formation is similar in molecular composition to vertebrate collagen type V.

Chondroitin sulphate Other important extracellular matrix molecules within vertebrate cartilages are the sulphated glycosaminoglycans, in particular chondroitin sulphates.

In vertebrate cartilage, the predominant chondroitin sulphates are chondroitin-4-sulphate (CS-A) and chondroitin-6-sulphate (CS-C) (Lash and Vasan 1983). A chondroitin sulphate similar to vertebrate chondroitin-6-sulphate has been found in polychaetes (Person 1983). The horseshoe crab (*Limulus*) branchial cartilage contains chondroitin sulphate 2,4-diS (CS-K) (Sugahara *et al.* 1996) [Sugahara *et al.* (1996) do not use the species name nor do they refer to the horseshoe crab, but refer to the King Crab, which is an out-dated common name for the horseshoe crab]. However, when the authors analysed the sulphation patterns, an additional sulphate group on the 3-C position was found that was undetectable following chondroitinase ABC digestion. Thus, *Limulus* CS appears to be tri-sulphated. Analysis of squid (*Illex*) CS-E by the same group of investigators revealed a similar tri-sulphated CS variety (Kinoshita *et al.* 1997). Falshaw *et al.* (2000) analysed the glycosaminoglycans of the squid *Nototodarus gouldi* and did not find any tri-sulphated CS. There were at least two varieties of CS in the cranial cartilage of the squid, a Ch4,6-diS (CS E) and a minor unsulphated (Ch0-S; CS) variety (Falshaw *et al.* 2000). An enzyme that transfers sulphate groups between the fourth and sixth positions and will interact with both CS-A and CS-B also exists in squid cartilage (Inoue *et al.* 1986; Ito and Habuchi 2000), possibly explaining the discrepancies in

CS type found by different research groups. It is clear from these studies that sulphation patterns of chondroitin sulphate molecules predominant in cartilage are variable across species. However, it is also clear that sulphation at the fourth or sixth position is the most common.

Core protein Chondroitin sulphate chains are linked to a core protein forming a proteoglycan. Core proteins within invertebrates appear to be different from any isolated from vertebrates. Vynios *et al*. (1985) showed that the protein core of the proteoglycan from the squid, *Illex illecebrous coidentii*, has a molecular weight of 150 kDa, and is high in threonine, serine, proline and glycine. Vynios and Tsiganos (1990) isolated three populations of proteoglycans from the squid *Illex* that differ in their protein core, the number of CS chains and the number and type of oligosaccharides. The ratio of galactosamine to uronic acid indicated the presence of proteoglycans other than chondroitin sulphate, suggesting that squid cranial cartilage contains high amounts of noncollagenous protein. Of the three populations, the proteoglycan D1D1A contains five CS chains on a 350-kDa protein core; D1D1B contains two or three CS chains on a 290-kDa protein core; and D1D2 contained two or three CS chains on a 260-kDa protein core (Vynios and Tsiganos 1990). This last fraction, D1D2, has been shown to interact with a squid link protein (Tsilemou *et al*. 1998). These proteoglycans are sensitive to degradation by elastase, and two of them (D1D2 and D1D1A) interact strongly with type I collagen (Vynios *et al*. 2000). This interaction is inhibited by degradation with either collagenase or chondroitinase ABC (Vynios *et al*. 2001).

Tsilemou *et al*. (1998) isolated a squid link protein, which interacts with aggrecan (a vertebrate CS-rich proteoglycan) and binds hyaluronic acid *in vitro*. Hyaluronic acid is not thought to be found in cephalopod cartilage, and in culture large amounts must be present to achieve binding (Tsilemou *et al*. 1998). However, proteoglycan staining of cartilages in the cuttlefish *Sepia officinalis* can be significantly reduced by predigestion of the cartilaginous matrix with hyaluronidase (A. G. Cole, personal observation), suggesting that small amounts of hyaluronic acid may be present in the cartilage of this species. Hyaluronic acid is a unique glycosaminoglycan because it does not form proteoglycans itself, but can interact with numerous other proteoglycans to form large aggregates of extracellular matrix material (Ayad *et al*. 1994). To date, hyaluronic acid has not been isolated from the cartilage of any invertebrate.

Additional extracellular matrix components Many components of vertebrate cartilage matrices have not been investigated in any invertebrate cartilage or chondroid connective tissue. These include small proteoglycans (e.g. decorin, biglycan, chondroadherin, and fibromodulin), cartilage oligomeric matrix protein and cartilage intermediate layer protein (Hedbom *et al*. 1992; Lorenzo *et al*. 1998). Cartilage oligomeric matrix protein and cartilage intermediate layer protein have been studied only in mammalian cartilage, and thus neither the specificity of the molecules to cartilage, nor their ubiquity within vertebrate cartilages is known.

Apart from the diversity of molecules yet to be investigated, the extracellular matrix of invertebrate cartilages contains analogues of all major matrix molecules found within vertebrate cartilages. As would be expected, given the phylogenetic distance between vertebrates and the different invertebrate clades, these matrix components are not identical. Divergence in both the biochemical structure and histological appearance of any tissue would be expected over a long period of time. The use of abundant amounts of type II collagen in cartilage is a major change that has occurred within the evolution of vertebrate cartilages.

Cartilage defined

Despite numerous attempts to classify vertebrate cartilage types based upon histological organization, relatively little attention has been given to the definition of cartilage in and of itself. In fact, most authors do not offer a strict definition of cartilage. To quote the authors of the first chapter of the first volume in the authoritative three volume series *Cartilage*, 'It is extremely difficult to define cartilage simply when attempting to encompass the complete spectrum of the types of this tissue existing at all ontogenetic states, in recent and fossil forms, throughout the vertebrates' (Moss and Moss-Salentijn 1983; p. 2).

Vertebrate researchers, who study model systems where the histological features of the cartilage conform to that considered typical of cartilage have little need for a precise definition of cartilage. Confusion as to what vertebrate cartilage may or may not be is only an issue for those who study skeletal tissues at the transition between cartilage and tendon or bone, for example, pathologists who may come across aberrant tissues that are intermediate in histology, or palaeohistologists who are interested in the relationships between skeletal tissues on an evolutionary time-scale (Hall 1978, 2002, 2004).

For those interested in cartilage and cartilage-like tissues in animals that fall outside the Vertebrata, a precise definition of cartilage is very important. Hall (1978) provides a definition of cartilage taking into account the hierarchical nature of cartilage as a tissue, and addressing each aspect of cartilage classification found within the vertebrate literature when he states: 'Cartilage is an avascular, supporting, and articular skeletal tissue (although like bone, it may arise ectopically outside the skeleton), deposited by both chondroblasts and by chondrocytes, and removed by chondroclasts. Its extracellular matrix, primarily composed of glycosaminoglycans, contains a smaller collagen component of type $[\alpha I(II)]_3$ (type II collagen). Cartilage may or may not exist as a mineralized tissue. Cartilage functions as the primary embryonic skeletal tissue in many parts of the embryo and as the articular tissue

at joints on both endochondral and membrane bones (in the latter case, the cartilage is known as secondary cartilage). Cartilage is found in both vertebrate and invertebrates.' (p. 7).

Although recognizing that cartilage can be found outside the vertebrate clade, Hall's definition shows strong biases towards vertebrate cartilage in two respects. There are no published studies on the development of invertebrate cartilages, therefore including references to embryonic function of cartilage and the classification of cartilages as 'secondary' apply only to vertebrates. Secondly, and perhaps more importantly, Hall includes the presence of type II collagen as diagnostic for cartilage. As we have seen, type II collagen is not unique to cartilage.

After spending the better part of two decades considering invertebrate cartilages, Philip Person came up with a general definition of cartilage. Person (1983) defines cartilage as: 'an animal tissue, usually endoskeletal, but also exoskeletal … Physically, cartilages are gristle-like, relatively rigid, and resistant to forces of compression, shearing, and tension. As a skeletal support structure, cartilage aids in locomotion and in resisting the force of gravity. Histologically, it is a form of connective tissue composed of polymorphic cells suspended in highly hydrated, metachromatic colloidal gel matrices of varying rigidity, composition, and abundance. Chemically, cartilage is characterized by its high content of collagen, glycosaminoglycan complexes, and water.' (pp. 33–34).

Person's definition was designed to be inclusive of all cartilage types in all animal groups, and as such is suitably generalized. In addition, Person and Mathews (1967) identified three criteria for the designation of cartilage:

(1) composed of cells suspended in a relatively rigid matrix of varying abundance;
(2) rich in acidic polysaccharides including chondroitin sulphates; and
(3) with high collagen content.

Re-defining cartilage

It is not possible to use the above criteria to unequivocally recognize, in histological section, a previously undescribed tissue as cartilage, such as that illustrated by Andersen *et al.* (2001) for the vestimentiferan *Riftia pachyptila*, where the fibroblastic nature of the cells rendered the authors unable to definitively answer their own question: 'Could the obturaculum of *Riftia* be considered as a primitive "cartilage"?' We propose that the aforementioned criteria be modified to reflect the fact that morphologically distinct chondrocytes need to be present, distinguishable from other mesenchymal connective tissue cells [in point (1) above]. Additionally, the term 'fibrous protein' should replace 'collagen' in point (3) above, to account for the lack of collagen in all lamprey cartilages (Wright *et al*. 2001) and type I and III cartilages of hagfish (Wright *et al.* 1998) and possibly arthropods, including *Limulus* (Wright *et al*. 2001) which do indeed have cartilaginous tissues. Our revised definition of cartilage, as modified from Person and Mathews (1967) and Person (1983) is as follows: Cartilage is a rigid animal connective tissue that functions by resisting shearing, tension, and compression, thereby providing skeletal support and/or protection for the animal. Histologically, cartilage is composed of large cells that are morphology distinct from other connective tissue cells in the animal; these cells are embedded within an extracellular matrix of varying abundance that has high amounts of fibrous protein (usually collagen or elastin) and ground substance (usually chondroitin sulphate).

Under this revised list of criteria, it would appear that the vestimentiferan obturaculum does not qualify as cartilage because it lacks distinct chondrocytes, although the fibroblasts may be unique in that they are surrounded by a basal lamina (Andersen *et al*. 2001).

Evolution of tissues

The presence of cartilaginous tissues among invertebrate taxa holds evolutionary significance, demanding that cartilage, as a tissue, appeared prior to the divergence and diversification of the vertebrates. This is strengthened by the fact that many similar types of molecules are used to build cartilage in both vertebrates and invertebrates. However, it could equally be argued that there is only one way to build cartilage, and the similarities in histology and biochemical properties between the various cartilaginous tissues indicate convergent evolution; for the interplay between convergence, homology and homoplasy see Hall (2003).

Recent attempts to examine the evolution of tissues address the evolution of specific gene products, the rationale being that molecular evolution of tissue-specific genes should directly reflect the evolution of the tissue (Miyata *et al*. 1994; Iwabe *et al*. 1996). Large-scale molecular analyses of many tissue-specific isoforms has indeed revealed a link between diversification of these different molecular isoforms and the associated radiation of vertebrate morphologies (Miyata *et al*. 1994; Iwabe *et al*. 1996).

This of course brings into question the relevance of deriving phylogenetic hypotheses at a level of analysis that differs from the data collected. The most familiar example of this problem is analysis of metazoan phylogeny, where investigators seek to determine the tree of life from the evolution of a single molecule, 18S rRNA (Field *et al*. 1988; Winnepenninckx *et al*. 1998). The relationship between molecularly derived hypotheses of gene evolution and the 'true' phylogenetic tree has been addressed by Page (2000) and Page and Charleston (1997) in what has been called *reconciliation tree reconstruction*, used to compare gene trees and a species tree or even to create a species tree from gene trees [Page (1998) has developed software, GENETREE, to create these reconciled trees, available as freeware from: http://taxonomy.zoology.gla.ac.uk/rod/genetree/genetree.html]. The basic premise behind this methodology is that there are often cases of duplication and subsequent divergence or loss

of many genes and gene products, making the creation of species trees from gene trees less straightforward than a 1 : 1 relationship. A reconciliation tree is the tree that is created to reflect these duplications and losses in the gene tree, resulting in a tree that reflects the true relationships, albeit with two identical trees that are connected at the node where duplication has occurred.

Genes and tissues

The relationship between genes and tissues is therefore not necessarily straightforward. OOta and Saitou (1999) categorized the relationship between gene duplications and the differentiation of tissues into three cases, only two of which are informative with regards to tissue evolution. The first case, which is noninformative about tissue evolution, concerns the duplication of a structural gene where a single regulatory region is retained. This is significant, but noninformative for our purposes, because the regulatory regions of the gene are thought to be responsible for tissue-specific expression of gene products (Arnosti 2003). When viewed as expression patterns in the animal, these paralogous genes show the same expression patterns. To be informative, a duplication of the regulatory region is required, either alone, or accompanied by duplication of the structural gene. Because of the change in the regulatory region, the expression pattern of the structural gene or paralogous gene pair may differ. Such changes in expression patterns are the connection between the evolution of the gene and the evolution of the tissue, allowing the latter to be inferred from the former (OOta and Saitou 1999).

An example: the evolution of muscle OOta and Saitou (1999) address the evolution of muscle tissues using molecular data to create gene trees of muscle-specific structural proteins, then superimposing these gene trees onto one another to create a cladogram depicting the evolution of muscle. This method is similar, if not identical, to the reconciliation methodology of Page (2000) and Page and Charleston (1997). Their results suggest that vertebrate muscle types form two distinct clades, one including skeletal and cardiac muscle, the other grouping smooth muscle with nonmuscle cells (OOta and Saitou 1999). Extending the analysis to nonvertebrate cell types revealed that arthropod skeletal muscle cells grouped with vertebrate skeletal and cardiac muscle, and nonmuscle grouped with nonmuscle in both groups (OOta and Saitou 1999). These results suggest that skeletal muscle, as a tissue type, evolved before the separation of vertebrate and arthropod lineages. That smooth muscle is more closely related to nonmuscle indicates that muscle, as a cell type, evolved more than once in the vertebrate lineage.

This study on muscle evolution may hold interesting parallels with the evolution of cartilage. Since cartilage is found in both vertebrates and invertebrates it suggests that cartilage may also predate the divergence of vertebrates and invertebrates. Furthermore, like muscle, the different types of vertebrate cartilage (e.g. hyaline cartilage, fibrocartilage, cell-rich cartilages) may have independent origins within the vertebrate lineage.

The origins of cartilage

Not all animals require cartilage as a structural tissue – there are other ways to solve similar structural problems – and therefore not all animals will be expected to have cartilage. For instance, one of the functions of cartilaginous endoskeletons is to antagonize musculature. In many invertebrates, such as annelids, an extensive hydro-skeleton provides resistance against which the muscles act. In contrast, all vertebrates have cartilage as a structural tissue – this solution is fixed in the vertebrate lineage, and as such, vertebrate cartilage, especially mammalian cartilage, should be considered as derived as any nonvertebrate cartilaginous tissue.

Evolution of cartilage

As cartilage and cartilage-like tissues are found in a variety of invertebrate taxa, cartilage, or at least the ability to form cartilage, either arose early in metazoan evolution or evolved more than once. After the origin of connective tissues the ability appeared to organize connective tissue extracellular matrix with cartilage-like properties (chondroid connective tissue), which would have conferred greater stiffness, a mechanical advantage for both embryos and adults that would have been selected for. Chondroid (cartilage-like) connective tissues must have preceded the evolution of vertebrate cartilage. It is very likely that the cartilaginous tissues of the different metazoan groups arose independently from this chondroid connective tissue. Thus the striking similarity between vertebrate cartilage and cephalopod cartilage is a result of convergence.

However, it is likely that the path taken by these different groups leading from chondroid connective tissue to cartilage is similar. In response to functional selective pressures, undifferentiated mesenchyme became regionally differentiated, with different concentrations of extracellular molecules. These selective pressures could include both skeletal and protective functions, the need to withstand compression and tension generated by musculature, and the need to protect a centralized nervous system. These, and other scenarios, are discussed by Beresford (1993) in the context of speculation of how invertebrate cells may have developed skeletal cell behaviours contributing to the evolution of the neural crest.

This regional differentiation of matrix composition results in the formation of a cartilage-like or chondroid connective tissue. Given time, and the persistence of the original functional selective pressures, the production of this regionally differentiated extracellular matrix could become ingrained

in the genetic architecture of the animal through regional specialization of the fibroblasts. These differentiated cells would be prechondrocytes.

The presumptive prechondrocyte is likely fibroblastic in morphology. Among vertebrates, it is common to find that presumptive chondroblasts have a fibroblast-like morphology. In fact, progenitor cells are often found within the perichondrium, where cells are fibroblastic in appearance. Chondroid connective tissue cells are also often fibroblastic, for example those described by Andersen *et al*. (2001) supporting the obteraculum of the vestimentiferan *Riftia pachyptila*. It can be envisioned that these presumptive chondrocytes, like all chondrocytes, required some cue from the external or extracellular environment to undergo this specialization, a cue such as induction by a mechanical stimulus on the extracellular matrix.

Within this matrix, the previously undifferentiated cells (fibroblasts) have undergone specialization into a novel cell type (chondrocyte), which produce the molecules of the extracellular matrix. In the course of this cellular specialization different phylogenetic lineages could have utilized different cell populations. In this case, chondrocytes may not be considered as homologous, but cartilages, as tissues derived from the same chondroid connective tissue precursor, are. The key concept that distinguishes cartilage from other chondroid connective tissues among vertebrates and invertebrates is that the cells that secrete the cartilage extracellular matrix are distinct from other connective tissue cells. Thus cartilage is both the extracellular matrix with fibrous protein and water absorbing polysaccharides, and the chondrocytes that secrete this rigid matrix.

Conclusions

The primary argument presented here is that cartilage is a metazoan tissue found in both vertebrates and invertebrates. It is important to note, however, that the term cartilage does not necessarily imply homology of tissues. Cartilage is defined structurally by the composition of its extracellular matrix and the presence of differentiated chondrocytes, both of which are distinct from other connective tissue cells and matrices. Because cartilage must be considered with reference to all forms found in all animal groups, new categories of cartilage are called for. We present one such category here, the vesicular cell-rich cartilage.

Furthermore, the ability to form chondroid connective tissue, with structural matrix properties similar to those seen in cartilage, arose before the divergence of vertebrates and invertebrates. Cartilage probably arose from this chondroid connective tissue independently in different lineages and thus the remarkable similarities between vertebrate and cephalopod cartilage are convergent. Further investigations into the many invertebrate cartilages and chondroid tissues will be required to fully elucidate this new view of cartilage a metazoan tissue.

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