# The Development and Evolution of Cartilage

**J Andrew Gillis,** University of Cambridge, Cambridge, United Kingdom



## Introduction: What is Cartilage, and Where is it Found Within the Vertebrate Skeleton?

Cartilage is an avascular, aneural skeletal tissue that contains cells (chondrocytes) embedded within an abundant extracellular matrix (ECM). Chondrocytes are generally round and reside in distinctive spaces (lacunae) within the ECM. Cartilage ECM is composed of chondrocyte-secreted collagen (predominantly type II collagen, encoded by the *col2a1* gene), proteoglycans (predominantly aggrecan, encoded by the *agc1* gene) and water, with fibrils of type II collagen entrapping hydrated proteoglycan aggregates to form a tissue with a smooth and glassy ('hyaline') appearance. Vertebrate cartilage is usually ensheathed by a fibrous layer of connective tissue called a perichondrium (**Fig. 1(a-c)**).

Vertebrate animals possess two distinct skeletal systems: An endoskeleton and a dermal skeleton (reviewed by Hall, 2005). These skeletal systems may be distinguished primarily by their modes of development, and both may involve cartilage to a greater or lesser extent. Below, I discuss where and how cartilage features in the vertebrate endo- and dermal skeleton, before reviewing molecular mechanisms that regulate cartilage development.

### 'Primary' Cartilage and the Vertebrate Endoskeleton

The vertebrate endoskeleton includes the axial (i.e., vertebral and rib) and appendicular (i.e., limb) skeletons, as well as some elements within the head (e.g., the braincase and ear ossicles). Endoskeletal elements form via a process known as endochondral ossification, whereby a skeletal element forms first as a cartilaginous template, with this template subsequently being replaced by bone (reviewed by Karsenty *et al.*, 2009) (**Fig. 2**). Cartilage that forms the template of endochondral bones is known as 'primary cartilage'.

Endoskeletal development begins with the accumulation of skeletal progenitor cells into a 'condensation' that roughly prefigures the shape of a presumptive skeletal element (Hall and Miyake, 2000) (**Fig. 2(a)**). These skeletal progenitor cells are mesenchymal, deriving from mesoderm (i.e., in the case of the axial or appendicular skeleton) or the neural crest (i.e., in the case of some elements within the head), and transiently express a distinct suite of cell adhesion molecules (e.g., N-cadherin and NCAM – Widelitz *et al.*, 1993; Oberlander and Tuan, 1994; Delise and Tuan, 2002a,b) and ECM proteins (e.g., fibronectin and type I collagen – Dessau *et al.*, 1980; Frenz *et al.*, 1989; Bang *et al.*, 2000) before overtly differentiating into cartilage. Cells in the centre of a mesenchymal condensation differentiate first into immature chondrocytes and begin to secrete type II collagen, and subsequent growth of the condensation proceeds both by the division of these immature chondrocytes and by expansion of ECM. Cells around the periphery of the condensation, on the other hand, continue to express type I collagen, and become the perichondrium (**Fig. 2(b)**).

Just prior to ossification, cartilage cells in the centre of a condensation undergo terminal differentiation, transitioning from immature to hypertrophic chondrocytes (**Fig. 2(c)**). This transition is marked by secretion of a change in cell morphology (hypertrophic chondrocytes become enlarged and more square-shaped), and by the secretion by hypertrophic chondrocytes of type X collagen (encoded by the *col10a1* gene) (Schmid and Linsenmayer, 1985; Kirsch and von der Mark, 1992). This zone of chondrocyte hypertrophy will form the primary centre of ossification, with blood vessels invading the cartilage, and transporting with them bone-forming cells (osteoblasts) which will replace the cartilage ECM with bone matrix (**Fig. 2(d)**). Cell lineage tracing studies in an ex vivo skeletal development model reveal that both endothelial cells of the invading vasculature and bone-forming osteoblasts derive from the perichondrium that surrounds the developing skeletal element (Colnot *et al.*, 2004). Upon ossification, the perichondrium becomes a periosteum, and will continue to generate new osteoblasts, facilitating appositional growth of the skeletal element. In most long bones, vascular invasion occurs again on either end of the condensation to form secondary centers of ossification, leaving zones of immature chondrocytes between the primary and secondary centers (**Fig. 2(e)**). The zone of cartilage between the primary and secondary centers of ossification is the epiphyseal (or growth) plate, and continued proliferation of chondrocytes within this zone contributes to the lengthening of the skeletal element. Upon cessation of growth, the epiphyseal plate ossifies (**Fig. 2(f)**).

It has long been thought that hypertrophic chondrocytes ultimately undergo programmed cell death (i.e., apoptosis) upon invasion of an ossification centre by osteoblasts. However, Roach *et al.* have argued, based on histological observations, that some hypertrophic chondrocytes undergo a final asymmetric cell division, with one daughter undergoing apoptosis, and the other differentiating into an osteogenic (i.e., bone-forming) cell (Roach, 1992; Roach *et al.*, 1995). More recently, genetic cell lineage tracing experiments have shown that many hypertrophic chondrocytes do, in fact, persist and differentiate into osteoblasts during embryonic development, growth and repair (Zhou *et al.*, 2014; Yang *et al.*, 2014; Park *et al.*, 2015; Hu *et al.*, 2017). For example, Yang *et al.* (2014) used genetic lineage tracing approaches to map the fate of *col10a1*-expressing hypertrophic chondrocytes in mice, and demonstrated that many of these cells give rise to type I collagen-expressing osteoblasts (and, eventually, osteocytes) in neonatal and adult bone. Similarly, Zhou *et al.* (2014) genetically labelled hypertrophic or immature chondrocytes using *col10a1*- and *agc1*-driver lines, respectively, in mice, and demonstrated that these cell types give rise to functional osteoblasts in normal

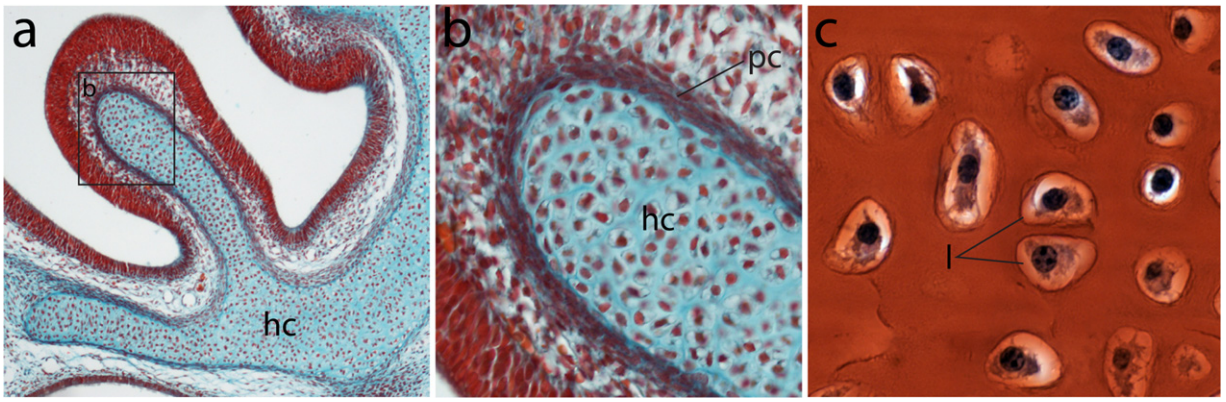


**Fig. 1** Histological features of vertebrate cartilage. (a) Hyaline cartilage (hc) in the developing braincase of an embryonic ferret (*Mustela putorius*). (b) Hyaline cartilage is often ensheathed by a fibrous perichondrium (pc). (c) Cartilage cells (chondrocytes) of the metapterygium (a basal fin cartilage) from a juvenile skate (*Leucoraja erinacea*) sit within lacunae (l), and are embedded in abundant extracellular matrix composed of type II collagen and aggrecan. (a) and (b) stained with Masson's trichrome. (c) stained with Safranin O.

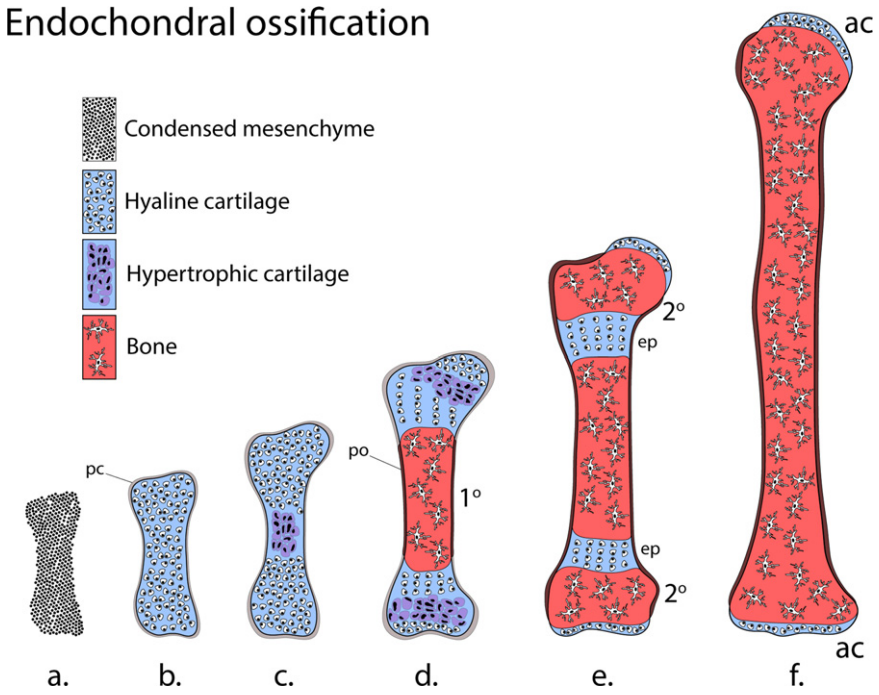


**Fig. 2** Simplified schematic representation of mammalian endochondral ossification, with an emphasis on the role of cartilage. (a) Mesenchymal cells condense at the site of skeletal development, before (b) differentiating into hyaline cartilage. At this stage, the cartilage element is ensheathed by a fibrous perichondrium (pc). (c) Chondrocytes in the centre of the cartilage enlarge to become hypertrophic chondrocytes. (d) Cartilage at the primary centre of ossification (1°) is replaced by bone, and the perichondrium becomes a periosteum (po). Chondrocytes become hypertrophic at either end of the element, and (e) are replaced by bone at secondary centers of ossification (2°). Epiphyseal plates (ep) of cartilage remain between the primary and secondary centers of ossification. (f) When growth ceases, the epiphyseal plates ossify, leaving only permanent articular cartilage (ac) at the ends of the element.

development and during bone fracture repair. These demonstrations of chondrocyte-to-osteoblast 'transdifferentiation' have led to a paradigm shift in the field of skeletal biology, and may have important implications for treatment of skeletal injuries (e.g., fracture repair) and degenerative joint disease (e.g., osteoarthritis). However, our understanding of the mechanisms underlying this phenomenon is still rudimentary.

While the vast majority of primary cartilage in the vertebrate endoskeleton is replaced by bone as described above, there are instances where this tissue persists as cartilage in the adult skeleton. One example of a persistent primary cartilage is Meckel's cartilage, a rod of hyaline cartilage that forms at the site of the future lower jaw, and that is subsequently engulfed by the dermal

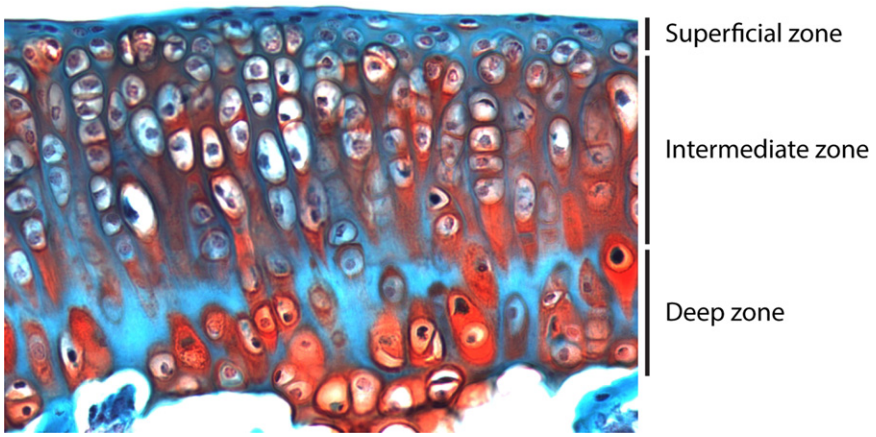


**Fig. 3** Mammalian articular cartilage. A Safranin-O/Fast Green-stained section through the proximal tibial articular cartilage of a 6 week-old mouse reveals the organization of chondrocytes into superficial, intermediate and deep zones. Image by Dr. Rebekah Decker, Genomics Institute of the Novartis Foundation.

bone(s) of the mandible. In both mammals and birds, the proximal end of Meckel's cartilage undergoes endochondral ossification, giving rise to middle ear ossicles (the malleus and incus) and the retroarticular process, respectively (Anthwal *et al.*, 2013; Hall, 2005). In mammals, much of the remainder of Meckel's cartilage is degraded through the action of cartilage-resorbing cells called chondroclasts (Anthwal *et al.*, 2017). However, in birds, Meckel's cartilage distal to the retroarticular process persists as a continuous rod of cartilage that traverses the lower jaw, and that provides additional skeletal support for the mandible. Another important example of persistent primary cartilage is articular cartilage, such as that found in the joints of endoskeletal long bones.

## Articular Cartilage

Additional zones of cartilage usually persist at the ends of developing long bones. This tissue will form the articular cartilage of the joint region, and unlike the epiphyseal plate, will remain cartilaginous indefinitely. Articular cartilage performs a vital buffering function, with the ECM providing a smooth, low-friction surface at sites of skeletal articulation. Aggrecan, the principal proteoglycan of cartilage matrix, consists of a protein core with covalently bound sulphated glycosaminoglycan (GAG) chains, and these GAGs retain water in order to maintain themselves in an optimally spaced configuration. Upon compression, water is squeezed from the matrix, and sulphated GAGs are brought into close contact with one another. Upon release of compression, repulsive interactions and the re-absorption of water returns GAGs to their optimally spaced configuration, returning the cartilage ECM to its turgid state (Roughley and Mort, 2014). The dynamic expulsion and re-absorption of water by cartilage matrix allows this tissue to act as an effective buffer against compressive forces.

Articular cartilage is organised into three zones, each with matrix and cellular properties reflecting specialised functional adaptations (Decker, 2017) (**Fig. 3**). The thin 'superficial zone' sits at the articular surface, and consists of specialised flattened chondrocytes that secrete glycoproteins, such as lubricin/proteoglycan 4 (encoded through alternative splicing of *Prg4* – Marcelino *et al.*, 1999; Jay *et al.*, 2001), to maintain frictionless motion between articulating bones. Beneath the superficial zone lies the 'intermediate zone', in which round chondrocytes are organised into stacks, and produce/maintain typical cartilage ECM products, such as type II collagen and aggrecan. The intermediate zone transitions into an underlying 'deep zone', which consists of stacks of larger, chondrocytes in a partially calcified ECM that grades into subchondral bone. This zonal architecture develops largely postnatally, in conjunction with the expansion of the articular cartilage by proliferation of progenitor cells in the superficial zone (and possible also in the deeper zone), through local cellular rearrangements, and through increases in cell size in the middle and deep zones (Mankin, 1962; Archer *et al.*, 1994; Hayes *et al.*, 2001; Dowthwaite *et al.*, 2003; Hunziker *et al.*, 2007; Williams *et al.*, 2010; Decker *et al.*, 2017). Evidence for the presence of persistent cartilage progenitor cells in adult articular cartilage is scant, and this – combined with the avascular nature of the tissue and the low turnover of ECM proteins – may account for why articular cartilage has such a limited capacity to heal spontaneously following injury (e.g., in osteoarthritis – Hunziker, 1999; Heinemeier *et al.*, 2016).

## 'Secondary' Cartilage and the Vertebrate Dermal Skeleton

The vertebrate dermal skeleton includes the plate-like bones of the skull, and, in reptiles and fishes, also includes various scales, scutes, denticles and fin rays. Dermal bone forms via a process known as intramembranous ossification, with mesenchymal condensations differentiating directly into bone without a cartilaginous template (Hall, 2005) (**Fig. 4(a-b)**). The cranial dermal skeleton is largely derived from the neural crest (Couly *et al.*, 1993; Chai *et al.*, 2000; Jiang *et al.*, 2002; Kague *et al.*, 2012), while postcranial dermal elements may be either mesoderm- or neural crest-derived (Kague *et al.*, 2012; Lee *et al.*, 2013; Mongera and

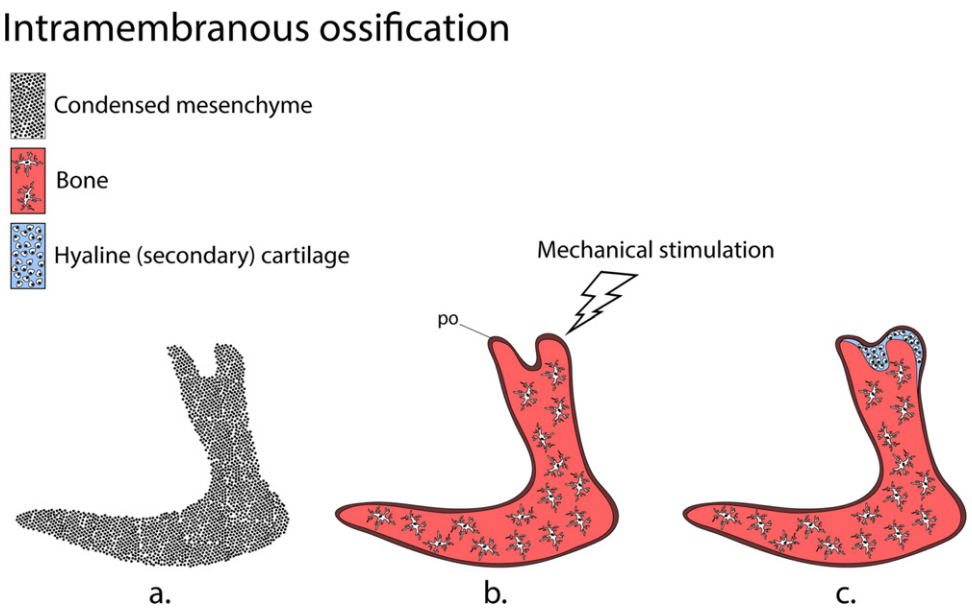


**Fig. 4** Simplified schematic representation of intramembranous ossification, and the development of secondary cartilage. (a) Mesenchymal cells condense at a site of skeletal development, before (b) differentiating directly into dermal bone. Dermal bones are surrounded by a fibrous periosteum (po). Mechanical stimulation of the periosteum (c) induces the differentiation of hyaline (secondary) cartilage.

Nüsslein-Volhard, 2013; Shimada *et al.*, 2013; Gillis *et al.*, 2017). Though dermal bones do not form via a cartilaginous intermediate, some of these elements nevertheless develop cartilage at sites of articulation or mechanical stress after osteogenesis has been initiated (**Fig. 4(c)**). Cartilages that develop in association with dermal bones are referred to as 'secondary cartilages' (Beresford, 1981).

Secondary cartilage has a patchy distribution across vertebrate phylogeny, having evolved independently at least three times (in mammals, birds and teleost fishes – reviewed in Hall, 2005). Examples of secondary cartilages include those that form on the mammalian and avian coronoid process (Anthwal *et al.*, 2008, 2015; Woronowicz *et al.*, 2018), on the avian quadratojugal and clavicle (Hall, 1967, 1968), and on various cranial dermal bones in a teleost (the black molly, *Poecilia sphenops* – Benjamin, 1989). Additionally, recent palaeohistological studies have found evidence of secondary cartilage on the cranial dermal bones of non-avian dinosaurs (Bailleul *et al.*, 2012, 2013). Secondary cartilage forms following differentiation of the dermal bones to which they are attached, and its differentiation is often dependent upon mechanical stimulation. In many cases, secondary cartilage originates within the periosteum of dermal bones, though in others it may arise as a sesamoid (i.e., as a small, nodular element that is distinct from its associated dermal bone). In the chick quadratojugal bone, secondary cartilage arises from the periosteum, and fails to form in the absence of articulation with the quadrate, but may be rescued in explanted quadratojugals by manual stimulation of the periosteum (Hall, 1967). Paralysis experiments have shown, in vivo, that secondary cartilage formation on the duck coronoid process is dependent upon embryonic movement (Woronowicz *et al.*, 2018). In the hamster, on the other hand, the secondary cartilage of the coronoid process initiates as a sesamoid, even in the absence of mechanical stimulation (Vinkka-Puhakka and Thesleff, 1993). However, this cartilage ultimately fuses with the condylar periosteum, and mechanical stimulus may be important for the maintenance of this tissue (Blackwood, 1966; Meikle, 1973). Secondary cartilage may persist as an articular-type cartilage, or may ultimately ossify, thus contributing to the growth of the bony element on which it formed (Hall, 2005).

Given their frequent origin from the periosteum of dermal bone – an otherwise osteogenic tissue – we may speculate that secondary cartilage derives from a specialised pool of cartilage progenitor cells that reside within this tissue. Alternatively, secondary cartilage could derive from uncommitted osteochondroprogenitor cells (i.e., a progenitor with the capacity to make both bone and cartilage), or through transdifferentiation of preosteoblast into chondrocytes. Recent, work has shown that the dentary bone of zebrafish regenerates via a cartilaginous intermediate, in stark contrast to its normal embryonic development by intramembranous ossification. Remarkably, during dentary repair, chondrocytes produce bone matrix, and likely originate from an osteogenic lineage within the dentary periosteum (Paul *et al.*, 2016). Thus, while definitive experiments testing whether secondary cartilage and associated dermal bone derive from common or distinct cell lineages are lacking, there is mounting evidence from both the dermal skeleton of fish and the endoskeleton of mammals (see previous section) that osteoblasts and chondrocytes may retain considerable plasticity upon differentiation, and an exceptional capacity to transdifferentiate from one cell type to another in response to environmental stimulus.

## Molecular Control of Vertebrate Cartilage Development

The progression of cartilage development (chondrogenesis) from mesenchymal condensation, through differentiation, to hypertrophy and ossification is regulated by signalling interactions and transcription factors acting on skeletogenic neural crest or

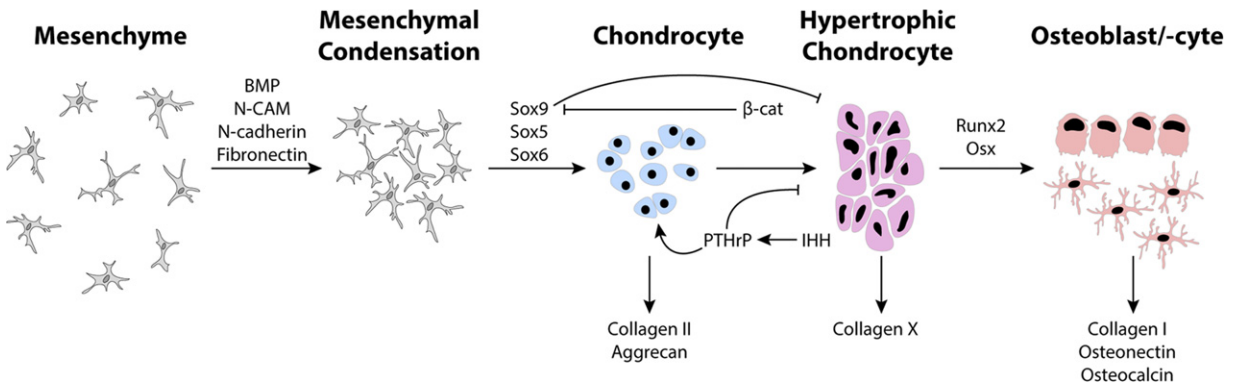


**Fig. 5** Summary of the molecular mechanisms of vertebrate cartilage development.

mesodermal mesenchyme. There are many examples of molecular mechanisms that pattern the shape and/or identity of specific cartilaginous elements within the vertebrate skeleton – e.g., the role of sonic hedgehog signalling in patterning tetrapod limbs (Riddle *et al.*, 1993), the role of the *Dlx* gene family in patterning the dorsoventral axis of the jaw (Depew *et al.*, 2002; Beverdam *et al.*, 2002) and the role of *Hox* genes in axial skeletal identity (Wellik, 2007). However, here, I will specifically review molecular mechanisms that may be general to the fundamental steps of cartilage development – i.e., the molecular basis of mesenchymal condensation, chondrocyte differentiation, proliferation and maturation. Molecular mechanisms outlined below are summarized in **Fig. 5**.

## BMP Signalling and Mesenchymal Condensation

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor beta (TGF-$\beta$) superfamily of signalling proteins. BMPs interact with cell surface receptors (the type I BMP receptors BMPR-1A, BMPR-1B, the type II BMP receptor BMPR-2, the type I activin receptor ActR-1A, or the type II activin receptors ActR-2A and ActR-2B), with intracellular transduction of BMP signals mediated by phosphorylation of receptor-associated SMAD transcription factors (SMAD-1, –5 or –8). When complexed with SMAD4, these factors are translocated to the nucleus, where they regulate transcription of downstream target genes (reviewed by Wang *et al.*, 2014). Recombinant TGF-$\beta$ ligands (including BMPs) are widely used as prochondrogenic factors in various in vitro chondrogenesis systems, reflecting a fundamental role for this superfamily in chondrogenesis in vivo.

Micromass culture (i.e., the high-density culture of dissociated primary limb bud mesenchymal cells) has been used to investigate chondrogenic differentiation in vitro, as these mesenchymal cells will spontaneously condense and differentiate into cartilaginous nodules in a manner that closely recapitulates cartilage development in vivo (Daniels *et al.*, 1996). Inhibition of BMP signalling in micromass culture by addition of the secreted BMP antagonist noggin leads to failure of mesenchymal condensation (Pizette and Niswander, 2000; Barna and Niswander, 2007). Furthermore, when wild type and *Smad4*-deficient mouse cells are co-cultured in micromass, *Smad4*-deficient cells are excluded from mesenchymal condensations (Lim *et al.*, 2015). Conversely, the addition of recombinant BMP2 to micromass cultures leads to qualitatively enhanced chondrogenesis (Pizette and Niswander, 2000). These findings point to an important role for BMP signalling in mesenchymal condensation in vitro.

BMP signalling has also been implicated in mesenchymal condensation in vivo. BMPR-1B is expressed in mouse limb bud preskeletal condensations, and deletion of BMPR-1B leads to reduced proliferation of condensed mesenchyme prior to chondrogenic differentiation (Yi *et al.*, 2000). Additionally, misexpression of noggin in chick limb buds inhibits mesenchymal condensation, leading to a loss of limb endoskeletal elements (Capdevila and Johnson, 1998; Pizette and Niswander, 2000). Finally, targeted genetic deletion of *Bmp2*, *Bmp4*, *Smad4*, or genes encoding BMP receptors (*BMPR-1A*, *BMPR-1B* and *ActR-1A*) from limb bud mesenchyme results in skeletal defects due to failed mesenchymal condensation (Bénazet *et al.*, 2012; Lim *et al.*, 2015; Bandyopadhyay *et al.*, 2006). Taken together, these studies point to BMP signalling as an important regulator of preskeletal mesenchymal condensation in limb buds. It should be noted, however, that genetic deletion of BMP signalling components alone, or in combination, does not impact all limb skeletal elements. It is therefore likely that there are spatially distinct requirements for BMP signalling during condensation of the limb skeleton, and additional factors regulating mesenchymal condensation that remain undiscovered.

## *Sox9* and *Sox5/6* Drive Cartilage Differentiation

Within mesenchymal condensations, the onset of cartilage differentiation is marked by the expression of *Sox9*. *Sox9* is a member of the SoxE class of high mobility group (HMG)-box family of transcription factors (reviewed by Kamachi and Kondoh, 2013), and

functions as a "master regulator" of chondrocyte differentiation. First indications that *Sox9* function was required for skeletal development came from the observation that patients with campomelic dysplasia (a rare genetic disorder caused by mutations in or near *Sox9*) present with both sex reversal and severe skeletal malformations (Foster *et al.*, 1994). It was subsequently discovered that *Sox9* is expressed in mesenchymal condensations during cartilage differentiation (Wright *et al.*, 1995), where it functions cell-autonomously to determine chondrocyte fate. In chimaeric mice, $Sox9^{-/-}$ cells are excluded from mesenchymal condensations and cartilage (Bi *et al.*, 1999), and conditional deletion of *Sox9* from limb bud mesenchyme and cranial neural crest cells results in complete loss of limb and head endoskeletal elements, respectively (Akiyama *et al.*, 2002; Mori-Akiyama *et al.*, 2003). Interestingly, deletion of *Sox9* after mesenchymal condensation leads to rapid maturation and hypertrophy of chondrocytes, indicating that *Sox9* may function not only in the initial differentiation of chondrocytes, but also in the maintenance of differentiated chondrocytes in an immature, proliferating state (Akiyama *et al.*, 2002).

It is now recognised that Sox9 drives chondrocyte differentiation through direct transcriptional regulation of the cartilage ECM molecules type II collagen and aggrecan. *Sox9* positively regulates the expression the SoxD-class transcription factors *Sox5/6*, and this Sox trio then co-operatively binds to cartilage-specific enhancers that drive expression of *col2a1* and *agc1* (Bell *et al.*, 1997; Lefebvre *et al.*, 1998, 2001; Smits *et al.*, 2001; Akiyama *et al.*, 2002; Han and Lefebvre, 2008). Interestingly, it has been reported that smad2/3 also complexes with Sox9 to enhance *col2a1* transcription in human mesenchymal stem cells (Furumatsu *et al.*, 2005). In light of the role of BMP signalling in mesenchymal condensation (see above), this association could reflect the link between TGF-$\beta$ signalling during mesenchymal condensation and subsequent Sox9-dependent cartilage differentiation.

### Wnt/$\beta$-Catenin Signalling Regulates Skeletal Cell Lineage Determination

The Wnt family of secreted glycoproteins is an important class of signalling molecules with a myriad of developmental functions, including axial patterning, morphogenesis and cell fate determination (reviewed by Nusse and Clevers, 2017). Briefly, upon binding of a Wnt ligand to a member of the frizzled family of cell surface receptors, Wnt signals may be transduced through one of three intracellular cascades: The canonical (i.e., Wnt/$\beta$-catenin) pathway, the planar cell polarity pathway, or the Wnt/$Ca^{2+}$ pathway. In the absence of Wnt signalling, cytoplasmic $\beta$-catenin is phosphorylated by a complex including glycogen synthase kinase 3 (GSK3) and targeted for degradation. However, upon activation of the canonical Wnt signalling pathway, GSK3 is sequestered to the membrane by intracellular components of the frizzled/LRP5/6 receptor complex. This leads to an accumulation of $\beta$-catenin in the cytoplasm, and the subsequent translocation of $\beta$-catenin to the nucleus, where it acts co-operatively with TCF/LEF transcription factors to drive the expression of downstream target genes.

Canonical Wnt/$\beta$-catenin signalling has been implicated as a negative regulator of chondrocyte differentiation in vivo and in vitro. Ryu *et al.* (2002) noted that expression of $\beta$-catenin is downregulated in mesenchymal condensations coincident with cartilage differentiation, both in the chick limb bud and in micromass cartilage nodules. They further demonstrated that enhanced accumulation of $\beta$-catenin by GSK3 inhibition prevented chondrocyte differentiation in vitro. A putative negative regulatory role for $\beta$-catenin in cartilage development was further corroborated by Akiyama *et al.* (2004), who demonstrated that conditional overexpression of a stabilised form of $\beta$-catenin in chondrocytes mirrors the *Sox9* loss-of-function phenotype, with a severe reduction of endoskeletal elements. Interestingly, conditional deletion of $\beta$-catenin from chondrocytes mirrors the effect of *Sox9* overexpression – i.e., failure of chondrocytes to hypertrophy and delayed onset of endochondral ossification. These observations, along with evidence that $\beta$-catenin and *Sox9* negatively regulate each other's transcriptional activities, have led to a model in which mesenchymal cell fate decisions are regulated by the opposing and reciprocally inhibitory activities of pro-chondrogenic *Sox9* and anti-chondrogenic Wnt/$\beta$-catenin signalling. It should be noted, however, that this model applies to primary cartilage, but not necessarily articular cartilage, where Wnt signalling promotes the differentiation of articular chondrocytes (see below).

Further studies have revealed that Wnt/$\beta$-catenin signalling also functions as a cell fate determinant at the point of osteogenic differentiation. Upregulation and nuclear localisation of $\beta$-catenin immediately precedes osteoblast differentiation in both endochondral and intramembranous ossification, and overactivation of Wnt signalling in differentiating cartilage may result in premature chondrocyte hypertrophy and enhanced ossification (Day *et al.*, 2005; Hill *et al.*, 2005). Conversely, conditional inactivation of $\beta$-catenin from mesenchymal condensations results in enhanced chondrogenesis, formation of ectopic cartilage, and (in developing dermal bones) the differentiation of chondrocytes in lieu of osteoblasts (Day *et al.*, 2005). Thus, Wnt/$\beta$-catenin signalling functions as an important regulator skeletal cell fate decisions at both early (chondrogenic) and later (osteogenic) stages of skeletal development.

### FGF, Indian Hedgehog and Parathyroid Hormone-Related Peptide Signalling Regulate Chondrocyte Proliferation and Hypertrophy

Following cartilage differentiation, the growth and shape of the final limb skeletal element is achieved through precise regulation of growth plate chondrocyte proliferation, maturation (e.g., hypertrophy) and ossification. Chondrocyte hypertrophy is marked by enlargement of chondrocytes and onset of type X collagen secretion, while osteogenic differentiation is marked by the expression in early osteoblasts of the transcription factors *Runx2* and *Osterix*, and by their secretion of characteristic bone matrix proteins (e.g., type I collagen, osteocalcin and osteonectin). Chondrocyte proliferation and maturation prior to endochondral ossification is

regulated largely by fibroblast growth factor (FGF), indian hedgehog (IHH) and parathyroid hormone-related peptide (PTHrP) signalling between growth plate cartilage and overlying perichondrium.

The FGFs are a family of secreted molecules that signal via four tyrosine kinase FGF receptors (FGFRs) (reviewed by Ornitz and Marie, 2002). During limb skeletal development, FGFR3 is expressed in immature chondrocytes, where it likely responds to FGF9 and FGF18 signals from the perichondrium (Peters *et al.*, 1992; Liu *et al.*, 2002; Ohbayashi *et al.*, 2002). Loss of FGFR3 function results in enhanced chondrocyte proliferation and endoskeletal overgrowth in mouse (Colvin *et al.*, 1996; Deng *et al.*, 1996). Conversely, activating mutations in FGFR3 are a cause of achondroplasia, the most common form of dwarfism, with shortened limbs resulting from reduced chondrocyte proliferation and premature ossification (Naski *et al.*, 1996; Lee *et al.*, 2017). Thus, contrary to the typical mitogenic (i.e., pro-proliferative) function of FGF signalling, endoskeletal FGFR3 signalling appears to function as a negative regulator of chondrocyte proliferation during endochondral ossification.

Hedgehog signals are transduced by interaction of a hedgehog ligand with patched (PTC) and smoothened (SMO) transmembrane co-receptors. Within growth plates, IHH is expressed in prehypertrophic and early hypertrophic chondrocytes, where it functions to maintain chondrocyte proliferation and inhibit chondrocyte hypertrophy (Vortkamp *et al.*, 1996). *Ihh*$^{-/-}$ mice form normal mesenchymal condensations, but ultimately develop shorted limbs due to reduced chondrocyte proliferation within the growth plate, and premature chondrocyte maturation and ossification (St-Jacques *et al.*, 1999). The pro-proliferative function of IHH signalling is thought to occur through direct signalling between hypertrophic and adjacent immature chondrocytes, as chondrocyte-specific deletion of *Smo* results in reduced proliferation, while overexpression of IHH or constitutively active SMO enhances chondrocyte proliferation (Long *et al.*, 2001).

The role of IHH signalling in regulating chondrocyte hypertrophy, on the other hand, occurs indirectly, via regulation of PTHrP signalling. PTHrP is expressed in perichondral tissue, while the PTHrP receptor (PTHrPR) is expressed in growth plate chondrocytes (at low levels in immature chondrocytes, and at higher levels in prehypertrophic and hypertrophic chondrocytes) (Vortkamp *et al.*, 1996). IHH is required for the maintenance of perichondral PTHrP expression, and as the *Ihh*$^{-/-}$ mouse, PTHrP$^{-/-}$ mice exhibit shortened limbs and premature hypertrophy of growth plate chondrocytes (Karaplis *et al.*, 1994). Thus, IHH and PTHrP are the molecular effectors of a feedback loop between hypertrophic chondrocytes, immature chondrocytes and the perichondrium, which functions to modulate chondrocyte proliferation and maturation within the growth plate.

### Molecular Control of Joint Formation

Synovial joints form at points of articulation within the appendicular skeleton (e.g., in limbs and digits). Joints are composed of thin pads of articular cartilage on the surface of each articulating element, encapsulated by a fluid-secreting joint capsule. Prospective articulating skeletal elements may form initially as a single, continuous condensation, which is subsequently fragmented to give rise to distinct bones. The development of endoskeletal joints is first visible as the formation of an interzone: A dense accumulation of flattened mesenchymal cells that sit at the future point of articulation between skeletal elements (reviewed by Salva and Merrill, 2017). Removal of the interzone leads to failure of joint formation and fusion of skeletal elements, indicating that this tissue is, itself, and important organiser of joint fate (Holder, 1977).

GDF5, another member of the TGF-$\beta$ superfamily of signalling proteins, plays a key role in the development of synovial joints in the vertebrate limb skeleton. GDF5 is expressed at sites of presumptive endoskeletal joints, both in the interzone and in surrounding tissues, prior to their appearance (Storm and Kingsley, 1996; Francis-West *et al.*, 1999). Cell lineage tracing experiments have shown that GDF5-expressing cells give rise to the vast majority of joint tissue (Shwartz *et al.*, 2016), and *GDF5*$^{-/-}$ mice lose most joints in their appendicular skeleton (Storm and Kingsley, 1996, 1999). Recent work has shown that articular cartilage develops largely from a distal proliferative zone (DPZ) of chondrocytes at the epiphyses of developing long bones (Ray *et al.*, 2015). The DPZ sits at an interface of BMP- and WNT-responsive tissues, with epiphyseal BMP signalling promoting the development of primary (i.e., epiphyseal plate) cartilage (Ray *et al.*, 2015), and interzone Wnt14 signalling promoting the development of articular cartilage (Hartmann and Tabin, 2001). Expression of the secreted BMP antagonist, *noggin*, becomes restricted to cells immediately adjacent to the DPZ, thus insulating these cells from epiphyseal BMP signalling and allowing them to respond to pro-articular Wnt signals from the interzone (Ray *et al.* (2015)). These studies illustrate the intricate molecular interactions that direct the development of cartilage subtypes, and how common signalling pathways (e.g., Wnt signalling) may perform distinct functions during different phases of skeletogenesis.

## The Evolution of Cartilage

All living vertebrates belong to one of three lineages: The osteichthyans (bony fishes, including tetrapods), the chondrichthyans (sharks, skates, rays and holocephalans) or the cyclostomes (lampreys and hagfishes). Osteichthyans and chondrichthyans, together, make up the jawed vertebrates (gnathostomes), while cyclostomes are the living jawless vertebrates. Cartilage was an integral component of the ancestral vertebrate skeleton. This may be inferred from the presence of cartilage in all living vertebrate groups, and from the preservation of cartilaginous skeletal elements in stem-vertebrate fossils (e.g., *Myllokunmingia* – Shu *et al.*, 1999, 2003; Hou *et al.*, 2002). Below, I briefly outline vertebrate cartilage diversity, and I then review the nature and distribution of invertebrate cartilage, in order to reconstruct the evolutionary history of this skeletal tissue.

### Diversity of Cartilage in Osteichthyans

While the hyaline cartilage described above (e.g., the primary cartilage of the mammalian endoskeleton) is generally regarded as "typical", a survey of vertebrate skeletal histology reveals a plethora of cartilage variants. For instance, mammals possess fibrocartilage (e.g., in menisci and the annulus fibrosus of intervertebral discs) and elastic cartilage (e.g., in the pinnae of the external ear), which exhibit unique mechanical properties owing to the inclusion of type I collagen and elastin fibres, respectively, within their ECM (Hall, 2005). Additionally, teleost fishes exhibit up to five 'classes' of cartilage-type tissues (Witten *et al.*, 2010), which span a continuum of ECM composition and cellularity, with tissues ranging from mammalian-like hyaline cartilage, to lipohyaline cartilage (i.e., cartilage containing a mixture of chondrocytes and adipocytes – Benjamin, 1990), to chondroid bone (i.e., a skeletal tissue composed of chondrocytes embedded in a bone-like matrix – Witten and Hall, 2002; Gillis *et al.*, 2006). Relatively little is known about the development and function of the diverse cartilage tissues of teleost fishes, though a greater understanding of these tissues may have important implications for both basic and clinical skeletal biology. Interestingly, many teleost cartilage types are reminiscent of tissues that occur pathologically in mammals (e.g., the chondroid bone that forms normally in the distal dentary of spawning Atlantic salmon could serve as a model for the hybrid bone- and cartilage-like tissues that occur in mammalian osteosarcomas – Witten and Hall, 2002). For a more complete review of the diversity of cartilage tissues in teleost fishes, see Witten and Hall (2015, 2019).

### The Chondrichthyan Endoskeleton

The adult endoskeleton of chondrichthyans is distinct from that of most other jawed vertebrates, in that it is composed almost entirely of hyaline cartilage. While chondrichthyans do possess some bone-like tissues (e.g., the neural arches and vertebral centra, which possess an ECM composed of type I collagen – Eames *et al.*, 2007; Enault *et al.*, 2015; Criswell *et al.*, 2017), the vast majority of their endoskeleton remains cartilaginous throughout life, and is not replaced by bone. As with other jawed vertebrates, the ECM of chondrichthyan cartilage is composed of type II collagen and aggrecan, though chondrocytes do not undergo hypertrophy, rendering the tissue most similar to the immature (i.e., non-hypertrophic) cartilage of birds and mammals. In light of the primitive presence of endochondral bone in jawed vertebrates (Donoghue *et al.*, 2006), the cartilaginous endoskeleton of chondrichthyans is likely a derived feature that arose through early premature arrest of the endochondral ossification pathway.

Chondrichthyans reinforce their endoskeleton through superficial mineralisation of the cartilage that sits immediately beneath the perichondrium (Dean and Summers, 2006). This mineralisation takes the form of "tesserae" – interconnected hexagonal plates that form a calcified rind around a skeletal element. Chondrichthyan tesserae consist of an "inner zone" containing typical chondrocytes embedded within a calcified matrix, and a "cap zone", which may derive from cells in the perichondrium. While it has been suggested that the chondrichthyan perichondrium could contain osteoblast like cells, and that the 'cap zone' of the tesserae could be bony in nature (Kemp and Westrin, 1979), the lack of chondrocyte hypertrophy and the unique cellular and growth features of the tesserae rather suggest that calcification of the endoskeleton in chondrichthyans represents an independent evolution of biomineral-based skeletal reinforcement (Dean *et al.*, 2009; Seidel *et al.*, 2017).

### Cyclostome Cartilage

Like chondrichthyans, cyclostomes possess a cartilaginous skeleton as adults, though the cyclostome cartilaginous skeleton reflects the retention of a primitive condition (and not a secondary loss of bone, as in chondrichthyans). The lamprey cartilaginous skeleton includes a braincase, gill basket, pericardium and rudimentary vertebral elements (Romer, 1970), and is composed of two broad types of cartilage: A cellular cartilage that makes up the majority of the skeleton, and that is generally reminiscent of hyaline cartilage (Wright *et al.*, 1988), and a 'mucocartilage', which surrounds the oral region (Johnels, 1948). Lamprey mucocartilage is, histologically, unlike typical hyaline cartilage, as the ECM has a more fluid-like consistency and contains mesenchymal cells in lieu of typical chondrocytes (Johnels, 1948; Wright and Youson, 1982).

Lamprey cartilage contains lamprin, a distinct class of non-collagenous ECM protein that shows some sequence homology with mammalian elastin (Robson *et al.*, 1993; Wright *et al.*, 1988). Based on the distinct biochemical composition of lamprey cartilage ECM, it was suggested that the last common ancestor of gnathostomes and cyclostomes lacked collagenous cartilage (Wright *et al.*, 2001), with a collagen-based cartilage ECM evolving along the gnathostome stem. However, it has since been shown that lampreys possess genes encoding type II collagen, and that these genes are co-expressed with a *SoxE* orthologue in developing cartilage (Zhang *et al.*, 2006). Further comparative gene expression analyses have also revealed that, despite their histological dissimilarities, lamprey mucoc- and gnathostome hyaline cartilages deploy a common gene regulatory network (Cattell *et al.*, 2011). These findings indicate that type II collagen-based cartilage is an ancestral feature of the vertebrate skeleton, and served as a foundation for the many cartilage variants that have arisen through vertebrate phylogeny.

### An Invertebrate Origin of Cartilage

Vertebrates are deuterostomes: A clade of animals that also includes the non-vertebrate chordates (urochordates and cephalochordates), as well as the echinoderms and hemichordates. Sister to the deuterostomes are the protostomes, a clade that comprises the ecdysozoans (e.g., arthropods, tardigrades, nematodes and priapulids) and the lophotrochozoans (e.g., molluscs, annelids, brachiopods, platyhelminthes and rotifers). Deuterostomes and protostomes are the two lineages of bilaterally

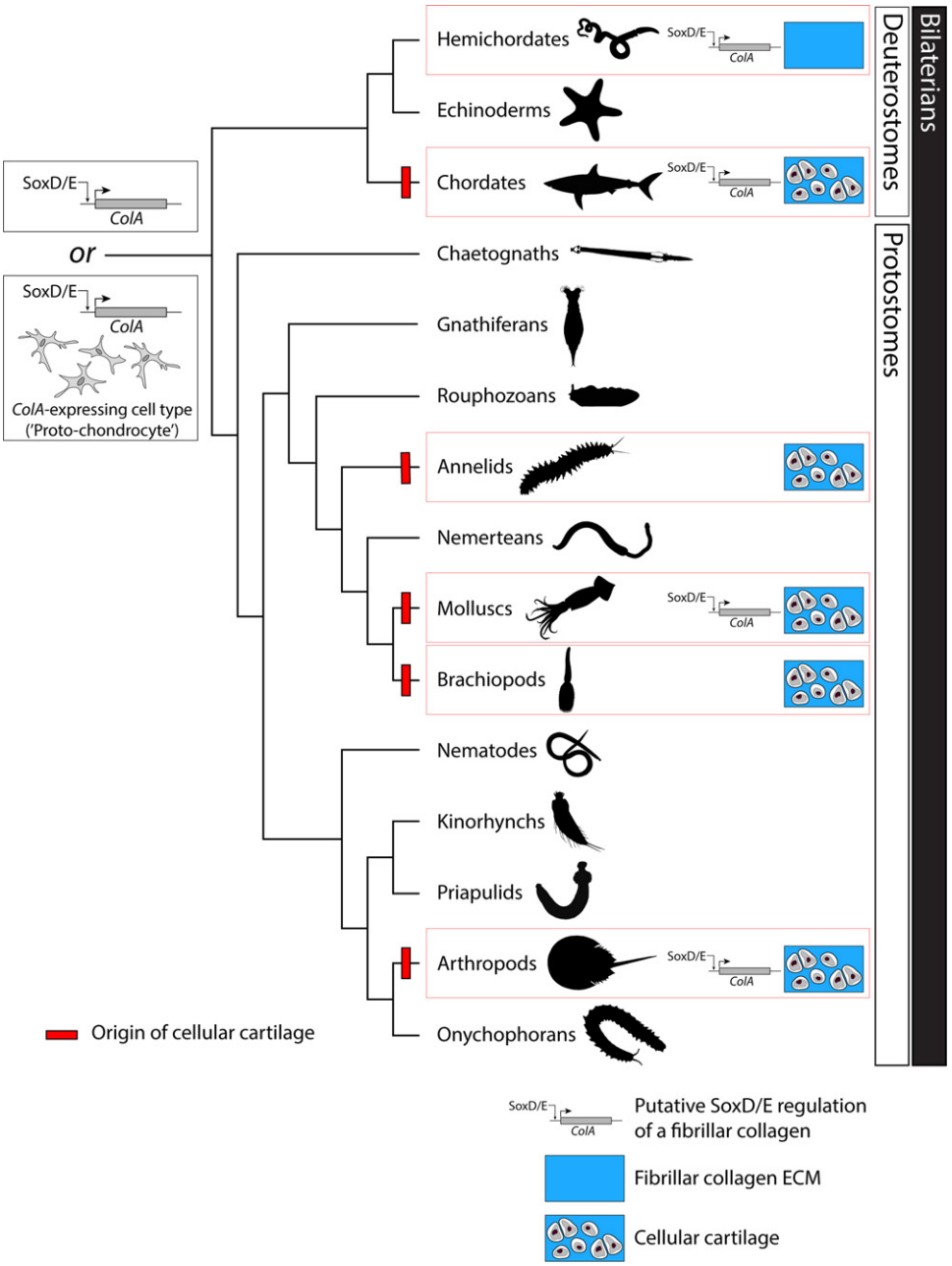


**Fig. 6** The evolution of cartilage in bilaterians. Cellular cartilage is present in chordates, annelids, molluscs, brachiopods and arthropods. In chordates, the gene encoding type II fibrillar collagen (*Col2a1*) is positively transcriptionally regulated by a SoxE-class (*Sox9*) and SoxD-class (*Sox5/6*) transcription factors. In cephalopod molluscs and horseshoe crabs, developing cartilage cells co-express SoxD/E genes and a class-A fibrillar collagen gene (*ColA*), indicating a possible regulatory relationship. The gill bar skeleton of hemichordates is composed of an acellular skeletal tissue that is composed of fibrillar collagen, and is secreted by epithelial cells that co-express *SoxE* and *ColA*. This distribution suggests that cartilage evolved independently at least five times in bilaterians. This may have occurred through the repeated co-option in distinct cell lineages of a deeply conserved *SoxD/E-ColA* gene regulatory network, or through the parallel evolution of cartilage from an ancient *SoxD/E-ColA*-expressing connective tissue cell type (a 'proto-chondrocyte') that was present in the last common ancestor of bilaterians.

symmetric animals (or "bilaterians") (reviewed by Telford *et al.*, 2015). A survey of endoskeletal tissues across bilaterians reveals that cartilage is not a uniquely vertebrate feature, but rather has a patchy distribution across non-vertebrate chordates, ecdysozoans and lophotrochozoans. Over the past several years, molecular analysis of various invertebrate cartilages or cartilage-like tissues have highlighted common developmental features, and point to the likely parallel evolution of cartilage via repeated deployment of a deeply conserved SoxE-collagen gene regulatory network.

Cephalochordates and hemichordates possess ciliated gill bars that are supported by an acellular collagenous skeletal tissue (Gillis *et al.*, 2012). This tissue is composed of fibrillar collagen, and is secreted by the *SoxE*-expressing endodermal cells that line

the pharyngeal gill bars (Rychel *et al.*, 2006; Rychel and Swalla, 2007). It has been proposed that the gill bar skeletal support tissue of cephalochordates and hemichordates could be a precursor of vertebrate cartilage, owing to their shared matrix composition, and their shared occurrence within homologous structures (i.e., the pharyngeal arches). However, given the acellular nature of the cephalochordate and hemichordate gill bar skeleton, it seems more likely that this tissue is an elaborated basement membrane. More recently, Jandzik *et al.* (2015) discovered that the oral cirri of cephalochordates are supported by cellular cartilage that co-expresses SoxE and SoxD transcription factors, as well as a gene encoding a clade-A fibrillar collagen (*ColA*). These findings firmly push the *SoxD/E*- and *ColA*-expressing chondrocyte back to the last common ancestor of chordates.

There are also many examples of cartilage in protostome invertebrates, including in the feeding tentacles of brachiopods and polychaete annelids, in the odontophore of gastropod molluscs, in the nuchal, funnel and optic skeletons of cephalopod molluscs, and in the endosternite and branchial skeleton of horseshoe crabs (Person and Matthews, 1967; Person and Philpott, 1969; Guralnick and Smith, 1999; Cole and Hall, 2004a,b; Katsuno and Sasaki, 2008; Golding *et al.*, 2009; Cole and Hall, 2009). These cartilages broadly resemble vertebrate cartilage in terms of ECM composition, contain rounded chondrocytes, and, in some cases (e.g., cephalopod molluscs), may be histologically indistinguishable from their vertebrate analogues (Cole and Hall, 2004a,b). Recently, molecular developmental analyses of cartilage development in the cuttlefish, *Sepia bandensis*, and the horseshoe crab, *Limulus polyphenus*, have highlighted even more striking parallels with vertebrate cartilage. Tarazona *et al.* (2016) have shown that *Sepia* and *Limulus* cartilages possess ECMs composed of collagen and proteoglycans, develop via pre-chondrogenic mesenchymal condensations, co-express SoxD/E transcription factors with genes encoding fibrillar collagen (*ColA*) and depend upon conserved signalling interactions that are shared with vertebrate cartilage.

It therefore appears as though cartilage has evolved independently at least five times in bilaterian animals (**Fig. 6**). Such convergent or parallel evolution may have occurred through the repeated, independent co-option in different cell lineages of an ancient *SoxD/E-ColA* gene regulatory network that was present in the last common ancestor of bilaterians. Alternatively, while cartilage itself is an unlikely primitive feature of bilaterians, a *ColA*-expressing connective tissue cell type (i.e., an evolutionary antecedent or 'latent homologue' of the chondrocyte – Stone and Hall, 2004) may have predated the divergence of deuterostomes and protostomes, and given rise to cartilage-like skeletal tissues, in parallel, in several bilaterian lineages (Brunet and Arendt, 2016). The latter is a hypothesis of cell type homology, which could be tested using a 'molecular fingerprinting' approach (Arendt, 2008). Molecular fingerprinting examines the unique gene expression features of cell types within a comparative phylogenetic framework, in order to infer the ancestral repertoire of cell types for a given clade. By using high-throughput sequencing technologies to characterise and compare a diversity of invertebrate skeletal and connective tissue cell types, it should be possible to reconstruct the evolutionary history and homology of cartilage-like cells and tissues through bilaterian evolution.

## Conclusions

Cartilage is a fundamental feature of the vertebrate skeleton, where it serves as a template for the endoskeleton, as a permanent tissue, or to resist mechanical stress at sites of endoskeletal and dermal skeletal articulation. The recent discovery that cartilage and bone cells have a capacity to transdifferentiate from one to the other is intriguing, and will have significant implications for our understanding of both normal skeletal development and disease. Additionally, while molecular mechanisms of normal cartilage development have been studied extensively in mammals, much remains to be learned about development of the full diversity of cartilage types that are found in non-mammalian vertebrates. Finally, studies of invertebrate cartilage highlight that this tissue has a very deep evolutionary origin, with certain features of cartilage possibly present in the last common ancestor of bilaterian animals. Further study of the phylogenetic distribution, embryonic origin and molecular development of invertebrate cartilage will allow us to rigorously test hypotheses of cartilage homology, and to disentangle the origin(s) of transcriptional mechanisms regulating fibrillar collagen expression and the origin(s) of the chondrocyte as a *bona fide* skeletal cell type.

## Acknowledgements

The author thanks Brian Hall, Kate Rawlinson, Katharine Criswell, Victoria Sleight and Jenaid Rees for helpful comments and discussion during the preparation of this review.

## References

Akiyama, H., Chaboissier, M.C., Martin, J.F., Schedl, A., de Crombrugghe, B., 2002. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes & Development 16, 2813–2828.

Akiyama, H., Lyons, J.P., Mori-Akiyama, Y., *et al.*, 2004. Interactions between Sox9 and beta-catenin control chondrocyte differentiation. Genes & Development 18, 1072–1087.

Anthwal, N., Chai, Y., Tucker, A.S., 2008. The role of transforming growth factor-beta signalling in the patterning of the proximal processes of the murine dentary. Developmental Dynamics 237, 1604–1613.

Anthwal, N., Joshi, L., Tucker, A.S., 2013. Evolution of the mammalian middle ear and jaw: Adaptations and novel structures. Journal of Anatomy 222, 147–160.

Anthwal, N., Peters, H., Tucker, A.S., 2015. Species-specific modifications of mandible shape reveal independent mechanisms for growth and initiation of the coronoid. EvoDevo 6, 35.

Anthwal, N., Urban, D.J., Luo, Z.-X., Sears, K.E., Tucker, A.S., 2017. Meckel's cartilage breakdown offers clues to mammalian middle ear evolution. Nature Ecology and Evolution 1, 0093.
Archer, C.W., Morrison, H., Pitsillides, A.A., 1994. Cellular aspects of the development of diarthrodial joints and articular cartilage. Journal of Anatomy 184, 447–456.
Arendt, D., 2008. The evolution of cell types in animals: Emerging principles from molecular studies. Nature Reviews Genetics 9, 868–882.
Bailleul, A.M., Hall, B.K., Horner, J.R., 2012. First evidence of dinosaurian secondary cartilage in the post-hatching skull of *Hypacrosaurus stebingeri* (Dinosauria, Ornithischia). PLOS One 7, e36112.
Bailleul, A.M., Hall, B.K., Horner, J.R., 2013. Secondary cartilage revealed in a non-avian dinosaur embryo. PLOS One 8, e56937.
Bandyopadhyay, A., Tsuji, K., Cox, K., *et al.*, 2006. Genetic analysis of the roles of BMP2, BMP4 and BMP7 in limb patterning and skeletogenesis. PLOS Genetics 2, e216.
Bang, O.-S., Kim, E.-J., Chung, J.G., *et al.*, 2000. Association of focal adhesion kinase with fibronectin and paxillin is required for precartilage condensation of chick mesenchymal cells. Biochemical and Biophysical Research Communications 278, 522–529.
Barna, M., Niswander, L., 2007. Visualization of cartilage formation: Insight into cellular properties of skeletal progenitors and chondrodysplasia syndromes. Developmental Cell 12, 931–941.
Bell, J.M., Leung, K.K., Wheatley, S.C., *et al.*, 1997. Sox9 directly regulates the type-II collagen gene. Nature Genetics 16, 174–178.
Bénazet, J.-D., Pignatti, E., Nugent, A., *et al.*, 2012. Smad4 is required to induce digit ray primordia and to initiate the aggregation and differentiation of chondrogenic progenitors in mouse limb buds. Development 139, 4250–4260.
Benjamin, M., 1989. The development of hyaline-cell cartilage in the head of the black molly, Poecilia sphenops. Evidence for secondary cartilage in a teleost. Journal of Anatomy 164, 145–154.
Benjamin, M., 1990. The cranial cartilages of teleosts and their classification. Journal of Anatomy 169, 153–172.
Beresford, W.A., 1981. Chondroid Bone, Secondary Cartilage and Metaplasia. Munich: Urban & Schwarzenberg.
Beverdam, A., Merlo, G.R., Paleari, L., *et al.*, 2002. Jaw transformation with gain of symmetry after Dlx5/Dlx6 inactivation: Mirror of the past? Genesis 34, 221–227.
Bi, W., Deng, J.M., Zhang, Z., Behringer, R.R., de Crombrugghe, B., 1999. Sox9 is required for cartilage formation. Nature Genetics 22, 85–89.
Blackwood, H.J.J., 1966. Growth of the mandibular condyle of the rat studied with tritiated thymidine. Archives of Oral Biology 11, 493–500.
Brunet, T., Arendt, D., 2016. Animal evolution: The hard problem of cartilage origins. Current Biology 26, R685–R688.
Capdevila, J., Johnson, R.L., 1998. Endogenous and ectopic expression of noggin suggests a conserved mechanism for regulation of BMP function during limb and somite patterning. Developmental Biology 197, 205–217.
Cattell, M., Lai, S., Cerny, R., Meulemans Medeiros, D., 2011. A new mechanistic scenario for the origin and evolution of vertebrate cartilage. PLOS One 6, e22474.
Chai, Y., Jiang, X., Ito, Y., *et al.*, 2000. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 127, 1671–1679.
Cole, A.G., Hall, B.K., 2004a. The nature and significance of invertebrate cartilages revisited: Distribution and histology of cartilage and cartilage-like tissues within the Metazoa. Zoology 107, 261–273.
Cole, A.G., Hall, B.K., 2004b. Cartilage is a metazoan tissue; integrating data from nonvertebrate sources. Acta Zoologica 85, 69–80.
Cole, A.G., Hall, B.K., 2009. Cartilage differentiation in cephalopod molluscs. Zoology 112, 2–15.
Colnot, C., Lu, C., Hu, D., Helms, J.A., 2004. Distinguishing the contributions of the perichondrium, cartilage, and vascular endothelium to skeletal development. Developmental Biology 269, 55–69.
Colvin, J.S., Bohne, B.A., Harding, G.W., McEwen, D.G., Ornitz, D.M., 1996. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nature Genetics 12, 390–397.
Couly, G.F., Coltey, P.M., LeDouarin, N.M., 1993. The triple origin of skull in higher vertebrates: A study in quail-chick chimeras. Development 117, 409–429.
Criswell, K.E., Coates, M.I., Gillis, J.A., 2017. Embryonic origin of the gnathostome vertebral column. Proceedings of the Royal Society B: Biological Sciences 284, 20172121.
Daniels, K., Reitler, R., Solursh, M., 1996. Micromass cultures of limb and other mesenchyme. Methods in Cell Biology 51, 237–247.
Day, T.F., Guo, X., Garrett-Beal, L., Yang, Y., 2005. Wnt/$\beta$-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Developmental Cell 8, 739–750.
Dean, M.N., Summers, A.P., 2006. Mineralized cartilage in the skeleton of chondrichthyan fishes. Zoology 109, 164–168.
Dean, M.N., Mull, C.G., Gorb, S.N., Summers, A.P., 2009. Ontogeny of the tessellated skeleton: Insight from skeletal growth of the round stingray Urobatis halleri. Journal of Anatomy 215, 227–239.
Decker, R.S., 2017. Articular cartilage and joint development from embryogenesis to adulthood. Seminars in Cell and Developmental Biology 62, 50–56.
Decker, R.S., Um, H.-B., Dyment, N.A., *et al.*, 2017. Cell origin, volume and arrangement are drivers of articular cartilage formation, morphogenesis and response to injury in mouse limbs. Developmental Biology 426, 56–68.
Delise, A.M., Tuan, R.S., 2002a. Analysis of N-cadherin function in limb mesenchymal chondrogenesis in vitro. Developmental Dynamics 225, 195–204.
Delise, A.M., Tuan, R.S., 2002b. Alterations in the spatiotemporal expression pattern and function of N-cadherin inhibit cellular condensation and chondrogenesis of limb mesenchymal cells in vitro. Journal of Cellular Biochemistry 87, 342–359.
Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A., Leder, P., 1996. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell 84, 911–921.
Depew, M.J., Lufkin, T., Rubenstein, J.L., 2002. Specification of jaw subdivisions by *Dlx* genes. Science 298, 381–385.
Dessau, W., von der Mark, H., von der Mark, K., Fischer, S., 1980. Changes in the patterns of collagen and fibronectin during limb-bud chondrogenesis. Journal of Embryology and Experimental Morphology 57, 51–60.
Donoghue, P.C.J., Sansom, I.J., Downs, J.P., 2006. Early evolution of vertebrate skeletal tissues and cellular interactions, and the canalization of skeletal development. Journal of Experimental Zoology (Molecular and Developmental Evolution) 206B, 278–294.
Dowthwaite, G.P., Bishop, J.C., Redman, S.N., *et al.*, 2003. The surface of articular cartilage contains a progenitor cell population. Journal of Cell Science 117, 889–897.
Eames, B.F., Allen, N., Young, J., *et al.*, 2007. Skeletogenesis in the swell shark *Cephaloscyllium ventriosum*. Journal of Anatomy 210, 542–554.
Enault, S., Munoz, D.N., Silva, W.T.A.F., *et al.*, 2015. Molecular footprinting of skeletal tissues in the catshark Scyliorhinus canicula and the clawed frog Xenopus tropicalis identifies conserved and derived features of vertebrate calcification. Frontiers in Genetics 6, 283.
Foster, J.W., Dominguez-Steglich, M.A., Guioli, S., *et al.*, 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 372, 525–530.
Francis-West, P.H., Abdelfattah, A., Chen, P., *et al.*, 1999. Mechanisms of GDF-5 action during skeletal development. Development 126, 1305–1315.
Frenz, D.A., Jaikaria, N.S., Newman, S.A., 1989. The mechanisms of precartilage mesenchymal condensation: A major role for interaction of the cell surface with the amino-terminal heparin-binding domain of fibronectin. Developmental Biology 136, 97–103.
Furumatsu, T., Tsuda, M., Taniguchi, N., Tajima, Y., Asahara, H., 2005. Smad3 induces chondrogenesis through the activation of Sox9 via CREB-binding protein/p300 recruitment. Journal of Biological Chemistry 280, 8343–8350.
Gillis, J.A., Fritzenwanker, J.H., Lowe, C.J., 2012. A stem-deuterostome origin of the vertebrate pharyngeal transcriptional network. Proceedings of the Royal Society B: Biological Sciences 279, 237–246.
Gillis, J.A., Alsema, E.C., Criswell, K.E., 2017. Trunk neural crest origin of dermal denticles in a cartilaginous fish. Proceedings of the National Academy of Sciences of the United States of America 114, 13200–13205.
Gillis, J.A., Witten, P.E., Hall, B.K., 2006. Chondroid bone and secondary cartilage contribute to apical dentary growth in juvenile Atlantic salmon. Journal of Fish Biology 68, 1133–1143.

Golding, R.E., Ponder, W.F., Byrne, M., 2009. Three-dimensional reconstruction of the odontophoral cartilages of Caenogastropoda (Mollusca: Gastropoda) using micro-CT: Morphology and phylogenetic significance. Journal of Morphology 270, 558–587.
Guralnick, R., Smith, L., 1999. Historical and biomechanical analysis of integration and dissociation in mollscan feeding, with special emphasis on the true limpets (Patellogastropoda: Gastropoda). Journal of Morphology 241, 175–195.
Hall, B.K., 1967. The formation of adventitious cartilage by membrane bones under the influence of mechanical stimulation applied in vitro. Life Sciences 6, 663–667.
Hall, B.K., 1968. In vitro studies on mechanical evocation of adventitious cartilage in chick. Journal of Experimental Zoology 168, 283–305.
Hall, B.K., 1986. The role of movement and tissue interactions in the development and growth of bones and secondary cartilage in the clavicle of the embryonic chick. Journal of Embryology and Experimental Morphology 93, 133–152.
Hall, B.K., 2005. Bones and Cartilage: Developmental and Evolutionary Skeletal Biology, first ed. London: Academic Press.
Hall, B.K., Miyake, T., 2000. All for one and one for all: Condensations and the initiation of skeletal development. Bioessays 22, 138–147.
Han, Y., Lefebvre, V., 2008. L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream promoter. Molecular Cell Biology 28, 4999–5013.
Hartmann, C., Tabin, C.J., 2001. Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. Cell 104, 341–351.
Hayes, A.J., MacPherson, S., Morrison, H., Dowthwaite, G., Archer, C.W., 2001. The development of articular cartilage: Evidence for an appositional growth mechanism. Anatomy and Embryology 203, 469–479.
Heinemeier, K.M., Schjerling, P., Heinemeier, J., *et al*., 2016. Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage. Science Translational Medicine 8, 346ra90.
Hill, T.P., Später, D., Taketo, M.M., Birchmeier, W., Hartmann, C., 2005. Canonical Wnt/$\beta$-catenin signaling prevents osteoblasts from differentiating into chondrocytes. Developmental Cell 8, 727–738.
Holder, N., 1977. An experimental investigation into the early development of the chick elbow joint. Journal of Embryology and Experimental Morphology 39, 115–127.
Hou, X.-G., Aldridge, R.J., Siveter, D.J., Siveter, D.J., Feng, X.-H., 2002. New evidence on the anatomy and phylogeny of the earliest vertebrates. Proceedings of the Royal Society B: Biological Sciences 269, 1865–1869.
Hu, D.P., Ferro, F., Yang, F., *et al*., 2017. Cartilage to bone transformation during fracture healing is coordinated by the invading vasculature and induction of the core pluripotency genes. Development 144, 221–234.
Hunziker, E.B., 1999. Articular cartilage repair: Are the intrinsic biological constraints undermining this process insuperable? Osteoarthritis and Cartilage 7, 15–28.
Hunziker, E.B., Kapfinger, E., Geiss, J., 2007. The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development. Osteoarthritis and Cartilage 15, 403–413.
Jandzik, D., Garnett, A.T., Square, T.A., *et al*., 2015. Evolution of the new vertebrate head by co-option of an ancient chordate skeletal tissue. Nature 518, 534–537.
Jay, G.D., Tantravahi, U., Britt, D.E., Barrach, H.J., Cha, C.J., 2001. Homology of lubricin and superficial zone protein (SZP): Products of megakaryocyte stimulating factor (MSF) gene expression by human synovial fibroblasts and articular chondrocytes localized to chromosome 1q25. Journal of Orthopaedic Research 19, 677–687.
Jiang, X., Iseki, S., Maxson, R.E., Sucov, H.M., Morriss-Kay, G.M., 2002. Tissue origins and interactions in the mammalian skull vault. Developmental Biology 241, 106–116.
Johnels, A.G., 1948. On the development and morphology of the skeleton of the head of Petromyzon. Acta Zoologica 29, 140–277.
Kague, E., Gallagher, M., Burke, S., *et al*., 2012. Skeletogenic fate of zebrafish cranial and trunk neural crest. PLOS One 7, e47394.
Kamachi, Y., Kondoh, H., 2013. Sox proteins: Regulators of cell fate specification and differentiation. Development 140, 4129–4144.
Karaplis, A.C., Luz, A., Glowacki, J., *et al*., 1994. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes & Development 8, 277–289.
Karsenty, G., Kronenberg, H.M., Settembre, C., 2009. Genetic control of bone formation. Annual Reviews of Cell and Developmental Biology 25, 629–648.
Katsuno, S., Sasaki, T., 2008. Comparative histology of radula-supporting structures in gastropods. Malacologia 50, 13–56.
Kemp, N.E., Westrin, S.K., 1979. Ultrastructure of calcified cartilage in the endoskeletal tesserae of sharks. Journal of Morphology 160.
Kirsch, T., von der Mark, K., 1992. Remodelling of collagen types I, II and X and calcification of human fetal cartilage. Bone and Mineral 18, 107–117.
Lee, R.T., Thiery, J.P., Carney, T.J., 2013. Dermal fin rays and scales derive from mesoderm, no neural crest. Current Biology 23, R336–R337.
Lee, Y.-C., Song, I.-W., Pai, Y.-J., Chen, S.-D., Chen, Y.-T., 2017. Knock-in human FGFR3 achondroplasia mutation as a mouse model for human skeletal dysplasia. Scientific Reports 7, 43220.
Lefebvre, V., Ping, L., de Crombrugghe, B., 1998. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO Journal 17, 5718–5733.
Lefebvre, V., Behringer, R.R., de Crombrugghe, B., 2001. L-Sox5 and Sox6 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis and Cartilage 9 (Suppl. A), S69–S75.
Lim, J., Tu, X., Choi, K., *et al*., 2015. BMP-SMAD4 signaling is required for precartilaginous mesenchymal condensation independent of Sox9 in the mouse. Developmental Biology 400, 132–138.
Liu, Z., Xu, J., Colvin, J.S., Ornitz, D.M., 2002. Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. Genes & Development 16, 859–869.
Long, F., Zhang, X.M., Karp, S., Yang, Y., McMahon, A.P., 2001. Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. Development 128, 5099–5108.
Mankin, H.J., 1962. Localization of tritiated thymidine in articular cartilage of rabbits. Journal of Bone and Joint Surgery 44, 688–698.
Marcelino, J., Carpten, J.D., Suwairi, W.M., Gutierrez, O.M., Schwartz, S., *et al*., 1999. CACP, encoding a secreted proteoglycan, is mutated in camptodactyly-arthropathy-coxa vara-pericarditis syndrome. Nature Genetics 23, 319–322.
Meikle, M.C., 1973. In vivo transplantation of the mandibular joint of the rat; An autoradiographic investigation into cellular changes at the condyle. Archives of Oral Biology 18, 1011–1020.
Mongera, A., Nüsslein-Volhard, C., 2013. Scales of fish arise from mesoderm. Current Biology 23, R338–R339.
Mori-Akiyama, Y., Akiyama, H., Rowitch, D.H., de Crombrugghe, B., 2003. Sox9 is required for determination of the chondrogenic cell lineage in cranial neural crest. Proceedings of the National Academy of Sciences of the United States of America 100, 9360–9365.
Naski, M.C., Wang, Q., Xu, J., Ornitz, D.M., 1996. Graded activation of fibroblast growth factor receptor 3 by mutations causing achondroplasia and thanatophoric dysplasia. Nature Genetics 13, 233–237.
Nusse, R., Clevers, H., 2017. Wnt/$\beta$-catenin signaling, disease and emerging therapeutic modalities. Cell 169, 985–999.
Oberlander, S.A., Tuan, R.S., 1994. Expression and functional involvement of N-cadherin in embryonic chondrogenesis. Development 120, 177–187.
Ohbayashi, N., Shibayama, M., Kurotaki, Y., *et al*., 2002. FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. Genes & Development 16, 870–879.
Ornitz, D.M., Marie, P.J., 2002. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes & Development 16, 1446–1465.
Park, J., Gebhardt, M., Golovchenko, S., *et al*., 2015. Dual pathways to endochondral osteoblasts: A novel chondrocyte-derived osteoprogenitor cell identified in hypertrophic cartilage. Biology Open 4, 608–621.
Paul, S., Schindler, S., Giovannone, D., *et al*., 2016. Ihha induces hybrid cartiage-bone cells during zebrafish jawbone regeneration. Development 143, 2066–2076.
Person, P., Matthews, M.B., 1967. Endoskeletal cartilage in a marine polychaete, *Eudistylia polymorpha*. Biological Bulletin 132, 244–252.
Person, P., Philpott, D.E., 1969. The nature and significance of invertebrate cartilages. Biological Reviews 44, 1–16.

Peters, K.G., Werner, S., Chen, G., Williams, L.T., 1992. Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. Development 114, 233–243.
Pizette, S., Niswander, L., 2000. BMPs are required at two steps of limb chondrogenesis: Formation of prechondrogenic condensations and their differentiation in chondrocytes. Developmental Biology 219, 237–249.
Ray, A., Singh, P.N., Sohaskey, M.L., Harland, R.M., Bandyopadhyay, A., 2015. Precise spatial restriction of BMP signaling is essential for articular cartilage differentiation. Development 142, 1169–1179.
Riddle, R.D., Johnson, R.L., Laufer, E., Tabin, C., 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75, 1401–1416.
Roach, H.I., 1992. Trans-differentiation of hypertrophic chondrocytes into cells capable of producing a mineralized bone matrix. Bone and Mineral 19, 1–20.
Roach, H.I., Erenpreisa, J., Aigner, T., 1995. Osteogenic differentiation of hypertrophic chondrocytes involves asymmetric cell divisions and apoptosis. Journal of Cell Biology 131, 483–494.
Robson, P., Wright, G.M., Sitarz, E., *et al.*, 1993. Characterization of lamprin, an unusual matrix protein from lamprey cartilage. Implications for evolution, structure, and assembly of elastin and other fibrillar proteins. Journal of Biological Chemistry 268, 1440–1447.
Romer, A.S., 1970. The Vertebrate Body, fourth ed. London: W.B. Saunders Company.
Roughley, P.J., Mort, J.S., 2014. The role of aggrecan in normal and osteoarthritic cartilage. Journal of Experimental Orthopaedics 1, 8.
Rychel, A.L., Swalla, B.J., 2007. Development and evolution of chordate cartilage. Journal of Experimental Zoology (Molecular and Developmental Evolution) 308B, 325–335.
Rychel, A.L., Smith, S.E., Shimamoto, S.T., Swalla, B.J., 2006. Evolution and development of the chordates: Collagen and pharyngeal cartilage. Molecular Biology and Evolution 23, 541–549.
Ryu, J.H., Kim, S.J., Kim, S.H., *et al.*, 2002. Regulation of the chondrocyte phenotype by beta-catenin. Development 129, 5541–5550.
Salva, J.E., Merrill, A.E., 2017. Signaling networks in joint development. Developmental Dynamics 246, 262–274.
Schmid, T.M., Linsenmayer, T.F., 1985. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. Journal of Cell Biology 100, 598–605.
Seidel, R., Blumer, M., Pechriggl, E.-J., *et al.*, 2017. Cartilage or bone? Collagens in the skeleton of "cartilaginous" fishes answer an old question. Journal of Structural Biology 200, 54–71.
Shimada, A., Kawanishi, T., Kaneko, T., *et al.*, 2013. Trunk exoskeleton in teleosts is mesodermal in origin. Nature Communications 4, 1639.
Shu, D.-G., Luo, H.-L., Conway Morris, S., *et al.*, 1999. Lower Cambrian vertebrates from south China. Nature 402, 42–46.
Shu, D.-G., Conway Morris, S., Han, J., *et al.*, 2003. Head and backbone of the Early Cambrian vertebrate Haikouichthys. Nature 421, 526–529.
Shwartz, Y., Viukov, S., Krief, S., Zelzer, E., 2016. Joint development involves a continuous influx of Gdf5-positive cells. Cell Reports 15, 2577–2587.
Smits, P., Li, P., Mandel, J., *et al.*, 2001. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. Developmental Cell 1, 277–290.
St-Jacques, B., Hammerschmidt, M., McMahon, A.P., 1999. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes & Development 13, 2072–2086.
Stone, J.R., Hall, B.K., 2004. Latent homologues for the neural crest as an evolutionary novelty. Evolution & Development 6, 123–129.
Storm, E.E., Kingsley, D.M., 1996. Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. Development 122, 3969–3979.
Storm, E.E., Kingsley, D.M., 1999. GDF5 coordinates bone and joint formation during digit development. Developmental Biology 209, 11–27.
Tarazona, O.A., Slota, L.A., Lopez, D.H., Zhang, G., Cohn, M.J., 2016. The genetic program for cartilage development has deep homology within Bilateria. Nature 533, 86–89.
Telford, M.J., Budd, G.E., Philippe, H., 2015. Phylogenomic insight into animal evolution. Current Biology 25, R876–R887.
Vinkka-Puhakka, H., Thesleff, I., 1993. Initiation of secondary cartilage in the mandible of the Syrian hamster in the absence of muscle function. Archives of Oral Biology 38, 49–54.
Vortkamp, A., Lee, K., Lanske, B., *et al.*, 1996. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273, 613–622.
Wang, R.N., Green, J., Wang, Z., *et al.*, 2014. Bone morphogenetic protein (BMP) signaling in development and human diseases. Genese & Diseases 1, 87–105.
Wellik, D.M., 2007. Hox patterning of the vertebrate axial skeleton. Developmental Dynamics 236, 2454–2463.
Widelitz, R.B., Jiang, T.-X., Murray, B.A., Chuong, C.-M., 1993. Adhesion molecules in skeletogenesis: II. Neural cell adhesion molecules mediate precartilaginous condensations and enhance chondrogenesis. Journal of Cellular Physiology 156, 399–411.
Williams, R., Khan, I.M., Richardson, K., *et al.*, 2010. Identification and clonal characterization of a progenitor cell sub-population in normal human articular cartilage. PLOS One 5, e13246.
Witten, P.E., Hall, B.K., 2002. Differentiation and growth of kype skeletal tissues in anadromous male Atlantic salmon (*Salmo salar*). International Journal of Developmental Biology 46, 719–730.
Witten, P.E., Hall, B.K., 2015. Teleost skeletal plasticity: Modulation, adaptation and remodelling. Copeia 103, 727–739.
Witten, P.E., Hall, B.K., 2019. Plasticity and variation of skeletal cells and tissues and the evolutionary development of actinopterygian fishes. In: Johanson, Z., Underwood, C., Richter, M. (Eds.), Evolution and Development of Fishes. Cambridge: Cambridge University Press, pp. 126–143.
Witten, P.E., Huysseune, A., Hall, B.K., 2010. A practical approach to the identification of the many cartilaginous tissues in teleost fish. Journal of Applied Ichthyology 26, 257–262.
Woronowicz, K.C., Gline, S.E., Herfet, S.T., Fields, A.J., Schneider, R.A., 2018. FGF and TGF$\beta$ signaling link form and function during jaw development and evolution. Developmental Biology. doi:10.1016/j.ydbio.2018.05.002.
Wright, E., Hargrave, M.R., Christiansen, J., *et al.*, 1995. The SRY-related gene Sox9 is expressed during chondrogenesis in mouse embryos. Nature Genetics 9, 15–20.
Wright, G.M., Youson, J.H., 1982. Ultrastructure of mucocartilage in the larval anadromous sea lamprey, *Petromyzon marinus* L. The American Journal of Anatomy 165, 39–51.
Wright, G.M., Keeley, F.W., Robson, P., 2001. The unusual cartilaginous tissues of jawless craniates, cephalochordates and invertebrates. Cell and Tissue Research 304, 165–174.
Wright, G.M., Armstrong, L.A., Jacques, A.M., Youson, J.H., 1988. Trabecular, nasal, branchial, and pericardial cartilages in the sea lamprey, *Petromyzon marinus*: Fine structure and immunohistochemical detection of elastin. The American Journal of Anatomy 182, 1–15.
Yang, L., Tsang, K.Y., Tang, H.C., Chan, D., Cheah, K.S., 2014. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. Proceedings of the National Academy of Sciences of the United States of America 111, 12097–12102.
Yi, S.E., Daluiski, A., Pederson, R., Rosen, V., Lyons, K.M., 2000. The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb. Development 127, 621–630.
Zhang, G., Miyamoto, M.M., Cohn, M.J., 2006. Lamprey type II collagen and Sox9 reveal an ancient origin of the vertebrate collagenous skeleton. Proceedings of the National Academy of Sciences of the United States of America 103, 3180–3185.
Zhou, X., von der Mark, K., Henry, S., *et al.*, 2014. Chondrocytes transdifferentiate into osteoblasts in endochondral bone during development, postnatal growth and fracture healing in mice. PLOS Genetics 10, e1004820.