Evolutionary repression of chondrogenic genes in the vertebrate osteoblast

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Abbreviations: *Bglap*, bone gamma-carboxyglutamic acid-containing protein (formerly *Osteocalcin*); *Col1a1*, collagen type I alpha 1 chain; *Col1a2*, collagen type I alpha 2 chain; Col2, collagen type II protein; *Col2a1*, collagen type II alpha 1 chain; *Col10a1*, collagen type X alpha 1 chain; *Ihh*, Indian hedgehog homolog; Mya, million years ago; RNA-seq, RNA sequencing; *Runx2*, runt-related transcription factor (formerly *Cbfa1*); *Sox9*, sex-determining region Y-box 9; *Spp1*, secreted phosphoprotein 1 (formerly *Osteopontin* or *bone sialoprotein I*)

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Abstract

Gene expression in extant animals might reveal how skeletal cells have evolved over the past 500 million years. The cells that make up cartilage (chondrocytes) and bone (osteoblasts) express many of the same genes, but they also have important molecular differences that allow us to distinguish them as separate cell types. For example, traditional studies of later-diverged vertebrates, like mouse and chick, defined the genes *Col2a1* and *Sox9* as cartilage-specific. However, recent studies have shown that osteoblasts of earlier-diverged vertebrates, such as frog, gar, and zebrafish, express these "chondrogenic" markers. In this review, we examine the resulting hypothesis that chondrogenic gene expression became repressed in osteoblasts over evolutionary time. The amphibian is an under-explored skeletal model that is uniquely positioned to address this hypothesis, especially given that it diverged when life transitioned from water to land. Given the relationship between phylogeny and ontogeny, a novel discovery for skeletal cell evolution might bolster our understanding of skeletal cell development.

Roll the clip, Jim: Phyletic constraint and skeletal cells

In cosmology, researchers observe distant light and leftover radiation from the Big Bang in an attempt to piece together the origins of the early universe. Evolutionary biologists take a similar approach by categorizing traits of living things, hoping to recreate the story of how life on Earth may have unfolded. Traditionally, bone and some cartilages were obvious targets for evolutionary study, because mineralization made them more likely to be retained in the fossil record [1]. Digging deeper into the evolutionary relationship of skeletal cells, however, a molecular fossil record of sorts can be unearthed from living animal models. To a great extent, this is possible due to phyletic constraint, which asserts that there are limitations on available evolutionary pathways in a given group of animals (i.e., a phylogenetic clade) [2]. As a result, each clade might retain some features that represent ancestral features of the last common ancestor with their sister clade. Accordingly, since amphibians diverged ~375 million years ago (Mya) from the last common ancestor of all tetrapods, they might exhibit better ancestral vertebrate features than mammals, who diverged from the last common ancestor of all amniotes more recently, ~310 Mya [3, 4]. Therefore, we might learn more

about the traits of ancestral vertebrates by studying earlier-diverged clades. In principle, phyletic constraint would leave enduring imprints that link living animals with the ancestors of their clade, specifically capturing in time features of an ancestral tetrapod, for example, in modern frogs. Combining phyletic constraint with advancements in high-throughput molecular techniques, it is feasible to quantitate the possible evolutionary history of skeletal cells in an unbiassed, systemic fashion. Let's play back the tape of skeletal cell evolution by comparing gene expression in various living animals.

The standard list of genes expressed in cartilage and bone came from studies in mouse and chick, two land animals that share a relatively-recent common ancestor (~310 Mya; [3]), compared to the evolutionary appearance of bone (~500 Mya; [5]). The cells that make up cartilage and bone are chondrocytes and osteoblasts, respectively (Fig. 1). Generally, the transcription factors Sox9 and Runx2 drive formation of chondrocytes and osteoblasts, respectively [6, 7]. In many contexts, it is useful to subdivide cartilage into two distinct forms: immature cartilage, made up of resting and proliferating chondrocytes (Fig. 1A); and mature cartilage, comprised of prehypertrophic and hypertrophic chondrocytes [6-8] (Fig. 1B). Immature cartilage is characterized by high levels of "typical" cartilage genes, such as *Sox9*, *Col2a1*, and *Aggrecan* [9-11] (Fig. 1A). While immature cartilage can be found throughout adults (e.g., the middle zone of articular cartilage; [1]), it often undergoes a series of maturation events (turning into mature cartilage) during the embryonic process of bone formation known as endochondral ossification [6-8, 12]. Mature cartilage is marked by *Ihh* and *Col10a1* expression (Fig. 1B), and its formation actually requires a coordinated downregulation of *Sox9* and upregulation of *Runx2* [6-8, 12-16]. Mature cartilage also can be found throughout adults (e.g., deep and calcified zones of articular cartilage; [6-8, 16]), but most mature cartilage is degraded during endochondral ossification [6-8, 17]. Since mature chondrocytes can express most known "bone" genes, including *Runx2, Spp1* (formerly called *Osteopontin*), and *Bglap* (formerly called *Osteocalcin*), only *Col1a1* and *Col1a2* are considered defining markers to discriminate osteoblasts from chondrocytes [18, 19] (Fig. 1C). Further illustrating the similarities between gene expression in mature chondrocytes and osteoblasts, some mature chondrocytes

actually transdifferentiate into osteoblasts [20-24]. On the other hand, *Col10a1* expression indeed distinguishes mature chondrocytes from osteoblasts in mouse and chick [6-8, 12-14, 16].

Osteoblasts suppressed chondrocyte genes during evolution

Recent studies have revealed that osteoblasts of earlier-diverged clades, like bony fishes and amphibians, express molecular markers normally associated with cartilage of later-diverged clades, such as mammals and birds [25-28]. A big surprise came when extremely high levels of *col10a1* expression (again, THE definitive marker of mature chondrocytes in chick and mouse) were demonstrated in osteoblasts of both zebrafish and gar [25]. Perhaps given the overlap in gene expression among mature chondrocytes and osteoblasts of chick and mouse, such a result was a relatively subtle variation among animal clades. However, even immature chondrocyte genes are expressed in osteoblasts of fish and frog. Low-to-moderate osteoblast expression of *col2a1*, which is usually only highly expressed in immature chondrocytes of chick and mouse, was demonstrated in zebrafish, gar, and even the western clawed frog, *Xenopus tropicalis* [25-28] (Fig. 2). As far back as 1988, often-overlooked papers described Col2 protein in fish bone [29-31]. Although most studies show near background *Col2a1* levels in bone of mouse and chick [e.g., 8], one study even showed relatively high *Col2a1* expression levels [32]. Col2 protein production in chick bones was not demonstrated, however, suggesting that evolutionary mechanisms of post-transcriptional regulation might also be at play. These unexpected data point out that any traditional understanding of the evolutionary relationship between the chondrocyte and osteoblast is based upon a biassed and incomplete molecular description of osteoblasts, since the vast majority of existing studies have focussed primarily on amniotes (e.g., mammals and birds). Therefore, any meaningful discussion about skeletal cell evolution needs to include all of the major vertebrate classes (Fig. 3), and despite some recent work, amphibians remain over-looked [3, 26-28, 35-50].

Since frogs and fish shared a common ancestor further back in evolutionary time than land animals, and phyletic constraint might preserve ancestral features, these data lead to the hypothesis that chondrocyte genes became repressed during evolution of the osteoblast (Fig. 3A). As a less parsimonious, alternative argument, bony fish and frogs could have independently converged on

increased chondrogenic expression in bone. Amphibians diverged from a common ancestor with mammals and birds approximately 375 Mya [3, 4] Given that most research is carried out on zebrafish, chick, and mouse, the intermediately-positioned frog provides a critical weigh station along any vertebrate evolutionary trajectory.

Using fingerprints to solve the hypothesis

High-throughput RNA sequencing (RNA-seq) is the unbiassed and quantitative method of choice for generating the comprehensive transcriptomic data needed to assess the levels of chondrocyte gene expression in osteoblasts [51, 52]. Rather creatively, the transcriptome of a specific cell type of interest has been termed its molecular fingerprint [53]. Similar to other traits, molecular fingerprints likely evolve through adaptation and constraint, but comparing molecular fingerprints is a novel approach for unraveling the evolution of cell types [2, 53, 54]. To evaluate the relationships among cell types, molecular fingerprints can be compared among different cell types in a given species (e.g., chondrocyte vs. osteoblast in mouse) or a given cell type in different species (e.g., osteoblasts in mouse vs. frog; Fig. 3). These analyses reveal not only qualitative data about what genes are included in each molecular fingerprint, but also quantitative data on the relative expression levels of genes expressed in both cell types. The latter aspect is critical in evaluating levels of chondrocyte gene expression during osteoblast evolution.

To test our osteoblast evolution hypothesis, several benchmarks might be used to determine how the osteoblast molecular fingerprint can be considered more or less chondrogenic (Fig. 4):

A. What percentage of genes from the osteoblast molecular fingerprint are considered classical chondrogenic markers (from the published chick and mouse literature)? How do these percentages vary across vertebrate clades?
B. What percentage of genes are shared between the osteoblast and chondrocyte molecular fingerprints within a vertebrate clade (immature and mature chondrocytes considered both separately and together)? How do these percentages vary across vertebrate clades?

C. Of shared genes between the osteoblast and chondrocyte, what are the relative levels of chondrocyte gene expression in the osteoblast? How do these levels vary across vertebrate clades?

Skeletal speculator

We conclude with a few further speculations. Two possible scenarios are consistent with the published data showing chondrocyte gene expression in osteoblasts of earlier-diverged vertebrates. First, repression of chondrocyte genes in osteoblasts might have occurred specifically in the ancestor to chick and mouse, perhaps related to adjustment to life in a strictly terrestrial environment. Second, this process might have been somewhat gradual during evolution of vertebrates. As amphibians, frogs are nicely positioned to resolve among these two possibilities. For example, if the frog osteoblast fingerprint were to present a chondrogenic level that falls somewhere between that of other established models, it would support the emergence of a gradual repressive pattern (Fig. 3A). Of course, the more animals analyzed, the better. For example, do cartilaginous fishes express even more chondrocyte genes in their bones than bony fishes (yes, we and others argue that living sharks and skates make bone; [55, 56])?

Expanding the relevance of this hypothesis, we suggest that an overlap in chondrocyte and osteoblast gene expression in earlier-diverged vertebrates provides insight into the evolutionary origins of the osteoblast. The fossil record clearly demonstrates that cartilage preceded bone, and chondrocytes and osteoblasts have similar functional and molecular features [57-59]. These observations led us to hypothesize that the osteoblast evolved from the chondrocyte [59]. Embryonically, both chondrocytes and osteoblasts develop from common progenitor cells—a nontrivial matter when establishing evolutionary connections between cell types [60, 61]. In fact, the idea that the first osteoblast evolved from a chondrocyte would be consistent with the fact that osteoblasts of earlier-diverged vertebrates express many chondrocyte genes.

Finally, we pay homage to Haeckel, de Beer, and others who noted the many similarities between development (ontogeny) and evolution (phylogeny; [62-64]). During endochondral ossification in mouse and zebrafish, at least a few of the cells that differentiate into immature

chondrocytes and transition to mature chondrocytes, eventually transdifferentiate into osteoblasts [20-24]. Does this recapitulate phylogeny? Interestingly, these developmental transitions involve the progressive downregulation of "typical" cartilage genes, such as *Sox9* and *Col2a1* [6-8, 12-15, 65] (Fig. 3B). Is this further insight into the evolution of the osteoblast? It would be fascinating to look at whether the changes to Sox9 binding loci during this developmental transition mirror those during evolution of the osteoblast (Fig. 3A). Nevertheless, skeletal cell evolution has been a longstanding topic of contention among researchers. Fortunately, comparing the molecular mechanisms underlying skeletal cell differentiation among extant vertebrate clades might provide us with the very clues needed to unravel the history of chondrocytes and osteoblasts.

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Figure legends

Figure 1| The relative location and molecular markers of major skeletal cell types during endochondral ossification. A schematic of a frog humerus illustrates where [A] resting and proliferating chondrocytes (red cells) are found in immature cartilage, relative to [B] prehypertrophic (green cells) and hypertrophic chondrocytes (yellow cells) of mature cartilage. The increase in cell size of maturing chondrocytes is made very apparent through Safranin O staining of sulfated proteoglycans in the cartilaginous extracellular matrix on tissue sections of a larval *Xenopus tropicalis* humerus [A vs. B]. [C] Osteoblasts (blue cells), located near invading vasculature (purple),

secrete tightly-wound collagen fibers into the bony extracellular matrix (e.g., blue perichondral bone), visualized with Aniline blue in Trichrome staining.

Figure 2| A new(old) vertebrate model for skeletal development: *Xenopus tropicalis*. [A] A ventral view of a stage NF64 *X. tropicalis* froglet stained with PTA (phosphotungstic acid) contrast agent and scanned at the Canadian Light Source, the only synchrotron in Canada, using phase-contrast imaging [33, 34]. [B] Ventral, [C] dorsal, and [D] lateral views of the craniofacial skeletal structures of a freshly metamorphosed, stage NF66 adult frog made visible through whole-mount Alcian blue/Alizarin red staining, where the blue indicates cartilage and red signifies calcified bone. Having diverged during a transitional period in evolution, the frog displays characteristics of both aquatic and terrestrial vertebrates, potentially making it a critical resource for understanding how evolutionary patterns in skeletal development may have arisen. Abbreviations: As = angulosplenial; Ch = ceratohyal; L = left; Mk = Meckel's cartilage; P = posterior; R = right; V = ventral.

Figure 3| Hypothetical evolution (and development?) of the osteoblast molecular fingerprint. [A] Molecular fingerprints can be compared across cell types and/or species to determine chondrogenic gene levels of osteoblasts. Comparing species, chondrogenic gene expression in osteoblasts of earlier-diverged vertebrates are relatively high compared to land vertebrates [25], suggesting that the vertebrate osteoblast may have evolved to become less chondrogenic. The frog osteoblast might have levels of chondrogenic genes that are somewhere in between osteoblasts of other aquatic vertebrates and land tetrapods, possibly revealing a gradual repression of this trait over evolutionary time. [B] Perhaps confirming further that ontogeny recapitulates phylogeny, a comparable chondrogenic downregulation is observed during the developmental process of endochondral ossification, when some maturing chondrocytes transdifferentiate into osteoblasts.

Figure 4| Comparing osteoblast molecular fingerprints across vertebrates to determine the levels of chondrogenic gene expression. Recent studies show that osteoblasts of earlier-diverged, aquatic clades express genes that are normally associated with cartilage (red) [25-28]. In contrast, terrestrial

osteoblasts express primarily “bone” genes (blue), thereby displaying little to no chondrogenic expression. RNA-seq of chondrocytes and osteoblasts in each vertebrate clade can reveal two important parameters to test the hypothesis that a gradual repression of chondrogenic genes occurred during evolution of the vertebrate osteoblast. First, an unbiassed list of the number of “cartilage” genes expressed in osteoblasts across vertebrates would be generated. Second, the levels of expression of any “cartilage” genes common to all osteoblast fingerprints would be determined (lighter shades of red).

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Author contributions

BFE and JKBN contributed to the conception of this study. JKBN stained the bone and cartilage in the specimens and generated all images except for Fig. 2A. JKBN and BFE wrote, read, revised, and approved the final manuscript.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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