

A REVIEW OF DIVERSITY IN THE EVOLUTION AND DEVELOPMENT OF CARTILAGE: THE SEARCH FOR THE ORIGIN OF THE CHONDROCYTE

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Abstract

Mammalian cartilage is a complex and developmentally important tissue type. Outside the mammalian lineage, cartilage may persist as an adult tissue, which shows a much wider diversity of histological structure. Tissues similar to vertebrate cartilage are also found within multiple invertebrate lineages, including mollusks, arthropods, and polychaetes, however the relationship of these tissues to vertebrate cartilage is unknown. Detailed molecular analysis of these invertebrate tissues is necessary to assess the degree of homology, if any, of cartilage throughout the metazoans. The purpose of the following review is to introduce readers to this diversity of cartilage and to synthesize the known genetic interactions that give rise to vertebrate cartilage into the format of a gene regulatory network (GRN). This chondrogenesis GRN highlights a large number of transcription factors known to be expressed during chondrogenesis, whose role in this process has yet to be elucidated. Verification and expansion of this initial GRN will assist in the identification of the core set of the genetic interactions necessary for the specification of the vertebrate chondrocyte. This is the necessary first step in allowing detailed comparison of the molecular signature of vertebrate chondrocytes with that of invertebrates with the ultimate goal of understanding the evolutionary origin of this important skeletal cell type.

Keywords: Gene regulatory network, molecular fingerprint, invertebrate cartilage, cell type evolution.

Introduction

Historically cartilage has been defined as a vertebrate tissue that forms part of the skeletal system that is grossly different from mineralized bone both in function and histological composition. Cartilage can be defined generally as an internal cellular support tissue high in fibrous protein and mucopolysaccharide content (see Cole and Hall, 2004a, for discussion). In mammals 3 classic types of cartilage have been histologically identified based upon the relative contribution and distribution of fibers within the extracellular matrix: hyaline cartilage is the typical cartilage that is generally referred to when one thinks of cartilage. Elastic cartilage contains additional elastin fibers, and fibrocartilage contains regions of organized fibrous tissue within the extracellular matrix. Developmentally, cartilage can be followed histologically through three general phases (Hall and Miyake, 2000). Prior to cell differentiation, within the mesenchyme a cellular condensation forms – known as the cartilage anlagen (Cameron *et al.*, 2010), protocartilage (Cole and Hall, 2009), or cartilage condensation (Hall and Miyake, 2000). These cells then begin to secrete the cartilage-specific matrix. As development progresses the chondrocytes continue to proliferate and generate extracellular matrix, which then calcifies as the cells enter the hypertrophic phase of differentiation. This calcified cartilage matrix is then replaced by bone – in a process known as endochondral bone formation. The purpose of this review is to introduce readers to the diversity that exists outside this general description of cartilage, and to synthesize what is known about the molecular nature of chondrocyte specification as a means to address the origin of this important cell type.

Diversity of Cartilage and Cartilage-like Tissues

Although hyaline cartilage is the most well-studied cartilaginous tissue both due to its developmental importance in laying down the scaffold for most of the major bones of the body, and due to the fact that cartilage is retained at the joint interfaces and is the primary tissue affected in arthritis – there also exists a large variety of histologically diverse cartilage types that persist as adult tissues, particularly found within fish lineages. Benjamin (1990) attempts to catalog this diversity, illustrating cartilages that vary in the relative amount of matrix with respect to cell density, and the presence of variation of components within the extracellular matrix. This system of cartilage classification has recently been re-visited (Witten *et al.*, 2010) wherein the authors stress that on

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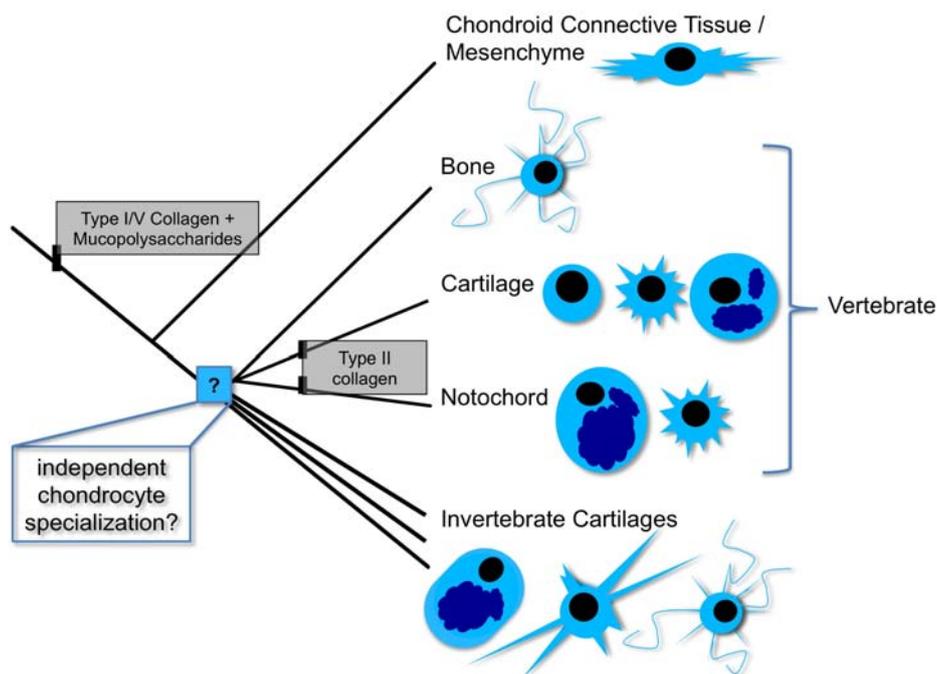


Fig. 1. Schematic depicting the problem the evolutionary relations between various connective tissues. Deposition of fibrous collagens and mucopolysaccharides in a structured connective tissue is an ancestral feature, whereas type II collagen is known to be specific to the vertebrate radiation and is present in both vertebrate cartilage and notochord. At present it is unclear whether the chondrocyte has evolved multiple times, or if there was a single origin and subsequent diversification of this cell type.

the basis of histology, cartilage is best considered as a spectrum of connective tissue types.

Interestingly, cartilage-like tissues have also been described in a variety of invertebrate lineages. Person and Philpot (1969) make the following statement regarding cartilage: “there is a body of information which suggest that just as nature knew how to form muscle, nerve, gland, and other tissues in the invertebrates, she also knew how to make cartilage, perhaps even before vertebrates appeared on the evolutionary scene” (pg. 2). The most compelling evidence to which the authors refer, is the histological organization of these invertebrate tissues. In fact within the invertebrates that have been investigated, a spectrum of histological tissues that parallels the diversity found within modern fishes is found (Cole and Hall, 2004b). Cartilages found within Cephalopod mollusks are remarkably similar to vertebrate hyaline cartilage at a histological level (Cole and Hall, 2004a). On close inspection, one can find one significant difference between vertebrate hyaline cartilage and that found within the cephalopod – the extra-cellular matrix of the cephalopod, in addition to the chondrocytes, is riddled with small canals – through which pass thin extensions of chondrocyte processes which retain connections between one another (Bairati *et al.*, 1998; Leone *et al.*, 2004; Cole and Hall, 2009). These cellular processes are a feature of vertebrate osteocytes rather than chondrocytes, although cell processes have been recently described within a subset of human chondrocytes using confocal microscopy to image live chondrocytes (Murray *et al.*, 2010), and chondrocytes

require a high density three dimensional culture environment to retain their spherical morphology (Abbott *et al.*, 1966); when cultured in two dimensions, chondrocytes revert to a fibroblast-like morphology and stop producing cartilage extracellular matrix (Chacko *et al.*, 1969). The physiological significance of these phenomena, if any, is currently unknown.

Another interesting feature of some cartilage-like tissues found within various invertebrate lineages is the presence of vacuolated cells (Cole and Hall, 2004b). For example, the polychaete family Sabellidae is defined by the presence of a cellular cartilage-like skeleton within its feeding tentacles (Kupriyanova and Rouse, 2008). This tissue is highly cellular, and the cells appear contain large vesicles (Cole and Hall, 2004b). Although hypertrophic chondrocytes are also characterized by an increase in cell volume that is accompanied by the accumulation of material within large vesicles, this is a histological feature that parallels the notochord in structure more so than the typical hyaline chondrocyte.

The vertebrate notochord produces many of the same extracellular matrix molecules as cartilage (for example Type II collagen), however at a much lower quantity, and the cells are large and vacuolated, surrounded by a mesenchymal-derived perinotochordal sheath. The affinity of the notochord to cartilage remains debatable; Cole and Hall (2004a) argue for its inclusion as a vesicular cartilage, whereas Witten *et al.* (2010) consider the notochord as “cartilage-related” (See Hall, 2005, for further discussion). Chondrocytes, or “chondrocyte-like” cells are predominant

in the mature nucleus pulposus (NP), which is derived from the embryonic notochord. Although historically the embryonically derived notochordal cells are considered to disappear and be replaced from invading cells of another lineage, evidence is growing that demonstrates a single origin of notochordal and NP cells, regardless of their histological appearance (reviewed in Risbud *et al.*, 2010). Kim *et al.* (2009) demonstrate the ability of NP cells to differentiate into three morphologically distinct phenotypes in culture, including both large vacuolated notochordal cells and the smaller chondrocyte-like cells, whereas Gilson *et al.* (2010) identify the presence of two seemingly distinct populations of cells within the mature NP, one of which continues to express notochordal markers. Similarly, there is circumstantial evidence that hypertrophic chondrocytes, rather than dying off as is generally presumed, have the capacity, and to at least some extent regularly undergo, proliferation and transdifferentiation into osteoblasts – changing their genetic program to express an osteogenic phenotype (reviewed in Wlodarski *et al.* 2006). Recently Hammond *et al.* (2009) identify a population of type II collagen expressing hypertrophic chondrocytes in the zebrafish that are differentially responsive to hedgehog signaling, and undergo a switch to an osteogenic phenotype, expressing osteoblast markers including *osterix*, *type I collagen*, and *osteocalcin*. Taken altogether, these data support the notion of a single skeletal cell origin and diversification towards a spectrum of cellular phenotypes. Combined with the fact that similar tissues are present in various invertebrate lineages begs the question: When exactly DID cartilage as a tissue type, or rather the chondrocyte as a cell type, evolve?

It is obvious from analysis of basal metazoan taxa that the capacity to synthesize fibrous protein and mucopolysaccharides in order to obtain a resistant connective tissue is a very ancient feature. However, perhaps the generation of a single cell type that is specialized for the local generation of this type of connective tissue has arisen independently multiple times – or perhaps only once with subsequent diversification (Fig. 1). The question becomes – how can we distinguish between these alternatives? The answer may lie in what has been described by Detlev Arendt as the Molecular Fingerprint of a cell type. The molecular fingerprint is the set of transcription factors, signaling molecules, and structural genes that are specific to a single cell type. Arendt (2003, 2005) defines a cell type as a “homogeneous population of cells expressing the same set of orthologous genes for specification and differentiation to implement a defined cellular phenotype”. Furthermore, “homologous cell types can be identified by molecular fingerprint comparisons” (Arendt, 2005; Arendt, 2008). Chondrogenesis is most often defined molecularly by the expression of the transcription factor Sox9 and the extracellular matrix molecule collagen type II, however neither of these genes are restricted to cartilage (Cheah *et al.*, 1991; Barrionuevo and Scherer, 2010). Thus, how might one define the molecular fingerprint for the chondrocyte?

Chondrocyte Molecular Fingerprint

Fig. 2 presents a synthesis of the transcriptional regulation of chondrogenesis in vertebrates, derived from data from the literature regarding regulatory interactions, which has been compiled into a gene regulatory network (GRN) framework. For simplicity, data has been divided into one of four phases corresponding to the developmental progression of chondrogenesis: Mesenchymal expression prior to cellular condensation, cellular condensation, differentiated cartilage, and hypertrophy. This division is arbitrarily over-simplified given the complexity of cartilage maturation, however serves as a necessary starting point to initiate the generation of this complex GRN. It is important to note, that most components of the network are derived from element-specific studies, and thus could reflect region specific patterning sub-modules that interact with the GRN rather than chondrogenesis *per se*, highlighting the importance of verifying the connectivity between each node. The HOX family of transcription factors has been largely omitted from the current analysis, as these genes are known to act as important patterning determinants and thus are not necessarily applicable to chondrogenesis as a whole. An exception is the Hoxc8 transcription factor, which has been shown to promote the expression of Ncam1 during pre-cartilage condensation, as well as promoting proliferation, and preventing progression to hypertrophy (Yueh *et al.*, 1998; Lei *et al.*, 2005) although the mechanism of this action remains undefined. A number of studies taking advantage of emerging technologies for looking at global gene expression changes by way of microarray data from micro-dissected tissues have been published in recent years (i.e., Yamane *et al.*, 2007; Belluccio *et al.*, 2008; Mienaltowski *et al.*, 2008; Zhang *et al.*, 2008; Shapiro *et al.*, 2009; Cameron *et al.*, 2009; Lui *et al.*, 2010). These studies have identified a number of transcription factors for which the role in chondrogenesis is unclear, included here as single-gene nodes to the left of the network. A number of these genes are introduced below.

The transcription factor Sox9 and type II collagen (*Col2a1*), the most abundant collagen within the cartilage extracellular matrix (ECM), are the two most widely studied components of the chondrogenesis GRN, as evidenced by the large number of inputs on both genes. Both osteocytes and chondrocytes arise from a common mesenchyme “stem” cell which are specified as osteochondro-progenitors through the action of the transcription factor Sox9; Sox9 has been demonstrated to be instrumental in the progression of these cells through the various phases of chondrocyte differentiation. In fact, Sox9 is the only transcription factor identified to date whose absence leads to the complete loss of all cartilage within the organism – thus highlighting its role as a sort of master-control gene for chondrogenesis. It is important to note that Sox9 is also expressed in many other tissues (testis: Barrionuevo and Scherer (2010); cerebral cortex: Alcock *et al.* (2009); gastric carcinoma: Zhou *et al.* (2010); pancreas: Rovira *et al.* (2010)) and thus although extremely

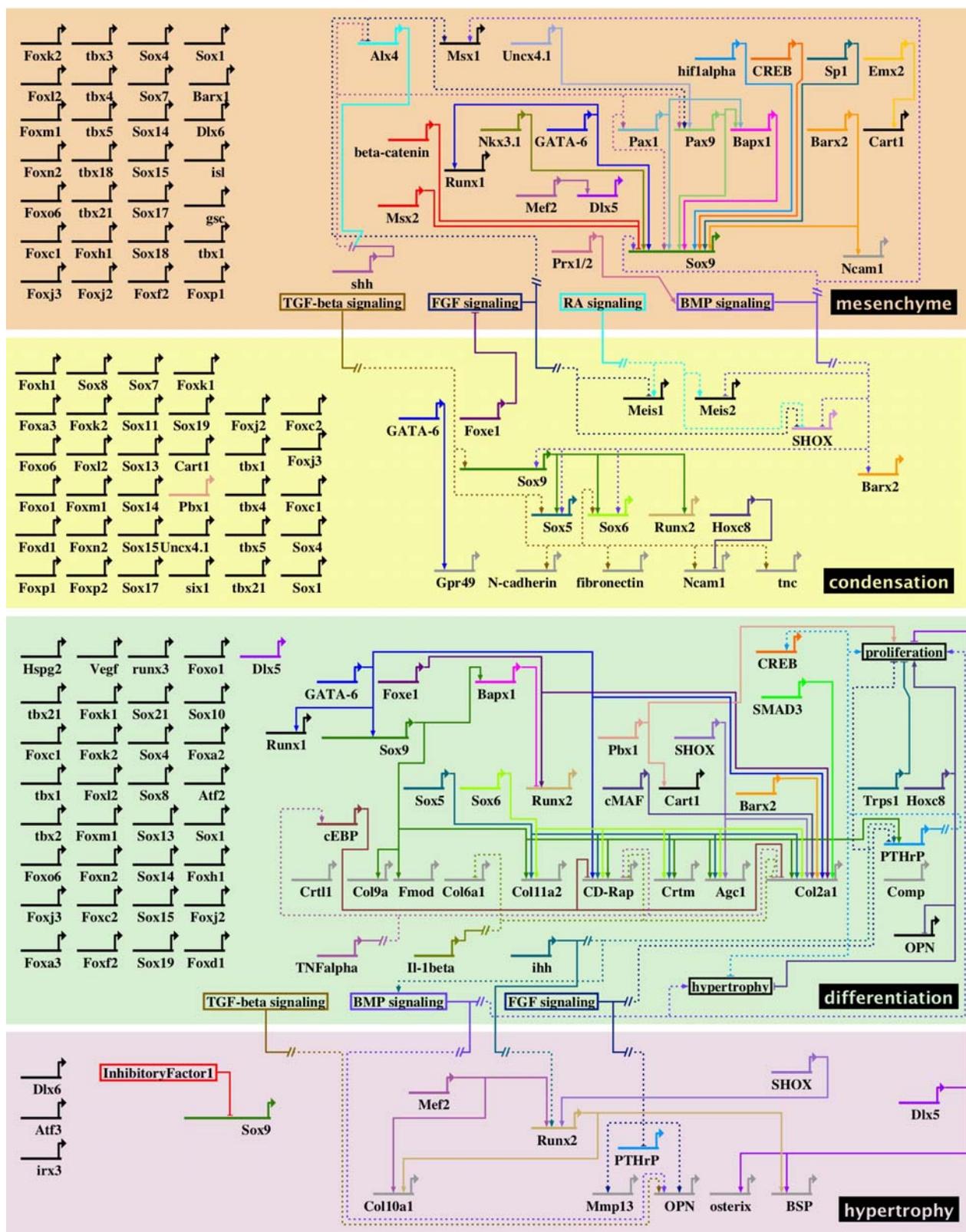


Fig. 2. GRN. Gene regulatory network of vertebrate chondrogenesis. Data regarding genetic interactions involved in the specification and differentiation of vertebrate chondrocytes compiled from published reviews and primary data papers was used as input to the GRN visualization program *BioTapestry*. Regulatory interactions are separated into four categories: mesenchyme (prior to cellular condensation); condensation; differentiation (onset of extracellular matrix deposition and maturation); hypertrophy (maturation and calcification of extracellular matrix prior to remodeling and bone deposition). Data derived from studies of mature cartilage, wherein expression data involves resting or proliferating chondrocytes, have been included in the differentiated cartilage category. Identification of novel transcription factors by way of comparative microarray studies are included as single-gene nodes to the left of

the network. Each interaction is shown as a line originating from the source transcription factor of the same color, connecting to the target gene(s). Only transcription factors and known differentiation products are included in the analysis; Signals originating from signaling pathways (BMP, Wnt, FGF, TGF signaling) are included only in the simplest form originating from the mesenchyme and/or during the differentiation phase, and are depicted as passing through a signaling cascade (//). Cell signaling derived interactions are depicted with dotted lines, and occur both within and between developmental phases. Arrowheads represent positive input to the target gene, whereas inhibitory interactions are depicted as $-|$ on the target gene. Differentiation factors are located near the bottom of the network and are depicted in grey. Genes active in multiple phases are present in each phase. Interactions depicted are not necessarily direct; in many cases this information is currently unknown.

important for chondrogenesis, is by no means specific for chondrogenesis *per se*. Nonetheless, Sox9 is commonly believed to be at the top of the hierarchy of this developmental program, which includes activating the expression of Sox 5, and Sox 6 (Akiyama *et al.*, 2002). Altogether these three transcription factors lead to the upregulation of cartilage matrix components including collagen 2 (*Col2a1*) and aggrecan (*Agc1*). A number of transcription factors and signaling pathways are involved in the activation of Sox9 prior to condensation and overt differentiation of the cartilage matrix, which is known to be blocked by nuclear beta-catenin (eg, Hill *et al.*, 2005). Sox9 is known to be down-regulated as chondrocytes pass into the hypertrophic phase of development, however the mechanism of this negative regulation is currently unknown (depicted here as “Inhibitory Factor 1”).

Cameron *et al.* (2009) published the first comprehensive study looking at the global gene expression profiles of early chondrogenesis in the mouse hind limb. The authors collected condensing mesenchymal cells, pre-cartilage condensations, and protocartilage cells, and subjected the RNA derived from each of these populations to a genomic microarray analysis to identify changes in gene expression as the mesenchymal cells undergo the transition to chondrocytes. This study revealed 29 transcription factors that were highly expressed in the early condensing mesenchyme, which were subsequently down regulated as the chondrocytes being to express their differentiated phenotype. Conversely, 11 transcription factors were upregulated during this transition (Cameron *et al.*, 2009, additional file 2). Of those expressed before overt differentiation of the chondrocytes, hence presumably active during chondrocyte specification, a number have already been identified as important in chondrocyte specification: *alx4* (Qu *et al.*, 1999), *barx2* (Jones *et al.*, 1997), *msx2* (Takahashi *et al.*, 2001) *pax9* (LeClair *et al.*, 1999), and *pbx1* (Selleri *et al.*, 2001). Interestingly, *Bapx1*, *Runx2*, *Runx3*, and *Dlx5*, are upregulated as the chondrocytes enter the differentiation phase (Cameron *et al.*, 2009, additional file 2), suggesting a role for these genes later in the gene regulatory network leading to cartilage formation. Novel transcription factors identified as up-regulated within the condensing mesenchyme include *gsc*, *isll*, *pitx1*, *six1*, *tf1*, *sox7*, *sox17* and eight members of the T-box (*tbx*) family of transcription factors, five of which show high levels of expression that are significantly down-regulated as the cells progress into the earliest stages of cartilage differentiation.

T-box transcription factors (*tbx*) have not been identified in any other microarray study wherein maturing cartilage has been the tissue examined, further suggesting that *tbx* genes may serve as negative regulators of chondrogenesis. *six1* was also identified as being highly expressed in surface articular cartilage cells with respect to more mature cartilage (Yamane *et al.*, 2007). *tf1* is known to be expressed in tissues immediately adjacent to developing tracheal cartilages (Park *et al.*, 2010); tissues harvested for the microarray study of Cameron *et al.* (2009) were taken by hand scraping, thus the possibility exists that contamination of non-chondrogenic mesenchyme is present in the analyzed RNA. This illustrates the importance of independent evaluation and validation of microarray data.

As expected from other studies that illustrate the role of Sox HMG transcription factors throughout chondrogenesis (Akiyama *et al.*, 2002; Akiyama, 2008), neither Sox9 nor Sox5 and Sox6 were shown to be differentially expressed during the condensation and onset of differentiation phases of cartilage formation, although expression levels of all three genes was elevated, in particular Sox 9 and Sox 6 (Cameron *et al.*, 2009). Other members of the Sox family previously known to be expressed during chondrogenesis include Sox 8, 10 (Chimal-Monroy *et al.*, 2003) and 13 (Wang *et al.*, 2006), part of the Sox E group of transcription factors. All three of these genes were demonstrated to be upregulated as the mesenchymal condensation passes into the initial phase of differentiation. Also identified as being highly expressed in the condensation phase of cartilage specification is the Sox C gene, Sox 11. Sox 11 was also identified as being highly expressed within the growth plate respect to the perichondrium and resting chondrocytes (Yamane *et al.*, 2007). The function of Sox 11 within chondrogenesis has not been identified, although this gene was identified in a microarray screen of osteoarthritic cartilage (Iliopoulos *et al.*, 2008). Interestingly, low levels of an additional seven Sox genes were found throughout chondrogenesis.

Another family of transcription factors identified is the forkhead-box (*fox*) family. *foxe1* is known to interfere with chondrogenesis in the zebrafish by negatively regulating FGF signaling by blocking the expression of the *fgfr3* receptor, as well as enhancing *Runx2* expression (Nakada *et al.*, 2009). The function of other *fox* genes in the context of chondrogenesis is unknown. However Cameron *et al.* (2009) find a number of these genes to be highly expressed during the condensation phase of chondrogenesis, with

foxa3, *foxo1*, and *foxd1* showing an increase in expression as the cells enter the differentiation phase. *Foxp1* showed slightly elevated expression during cartilage condensation, and was also identified by Yamane *et al.* (2007) as showing a 6 fold increase in expression within surface articular chondrocytes with respect to the growth plate chondrocytes.

It is evident from the number of previously unreported transcription factors represented in Fig. 2, that much remains to be discovered regarding the gene regulatory network for chondrogenesis in vertebrates, in particular to unravel the minimal regulatory kernels (Davidson and Erwin, 2006; Nowick and Stubbs, 2010) that may represent the ancestral molecular fingerprint for the chondrocyte. The large phenotypic overlap between various vertebrate and invertebrate skeletal cells compounds this problem and the question of the evolutionary origin of these cell types remains (Fig. 1). There are at least three possibilities: that chondrocytes are homologous across lineages and thus cartilage is a homologous tissue. That some chondrocytes in some lineages are homologous. Or that all chondrocytes in different lineages are the result of independent, convergent evolution. To distinguish between these alternatives, detailed molecular analyses of non-vertebrate cartilages is required.

Molecular Studies of Invertebrate Cartilages

These types of analyses have been carried out independently by various groups, leading to somewhat different conclusions. Wada (2010) recently pulled together an analysis of deuterostome genomic information to conclude that at least within the deuterostomes there is strong evidence for an ancestral gene regulatory network where Sox genes regulate the expression of fibrous collagens. Both SoxE and Runx homologs are present within the cellular cartilaginous skeletons of non-vertebrate chordates (lamprey and hagfish: McCauley and Bronner-Fraser, 2006; Zhang *et al.*, 2006; Ohtani *et al.*, 2008; Hecht *et al.*, 2008). Presently the only non-chordate invertebrates to be investigated have only an acellular branchial skeleton. Although SoxE was found to be expressed within the hemichordate skeleton (Rychel and Swalla, 2006), it is absent within the skeletal tissues of the cephalochordate, while present within the notochord (Meulemans and Bronner-Fraser, 2007) as is the Runx gene (Hecht *et al.*, 2008). The absence of the chondrocyte in these systems precludes robust conclusions regarding the ancestral nature of a chondrocyte gene regulatory network; given the ease with which things can be lost over time versus the challenges in re-inventing the wheel, so to speak, we might conclude that absence of evidence is by no means evidence of absence.

The invertebrate system that may be the most adapt for addressing the question of the evolutionary origins of the chondrocyte as a cell type is the European cuttlefish, *Sepia officinalis*. This animal has 13 different skeletal elements that have been described as cartilaginous. Of these, histological analysis reveals that more than half of

them correspond histologically to hyaline cartilage (Cole and Hall, 2009). The development of these cartilaginous elements is somewhat variable despite the lack of differences in the histology of the mature tissue: Most of these cartilages develop as a metaplasia from uncondensed mesenchymal tissue, however a number of the more prominent, and phylogenetically conserved amongst cephalopod lineages, go through a condensation phase similar to that described for vertebrates. And of these, a number of elements develop from a condensation that directly underlies a cuboidal epithelial layer. The *sepi* hedgehog ortholog is expressed in association with some cartilaginous tissues (Navet *et al.*, 2009) in particular within the overlying cuboidal epithelial layer prior to the overt differentiation of the underlying cartilage (L. Focareta and A.G. Cole, unpublished data), suggesting that this important signaling molecule may be involved in the early specification and/or patterning of cuttlefish cartilage formation. A detailed molecular analysis of this cuttlefish cartilage is currently underway with the goal of addressing the level of homology, if any, that exists between cellular cartilage from an invertebrate model and vertebrate cartilage.

Perspectives

Cartilage is a complex tissue type, whose complexity is enhanced by the existence of numerous developmental phases and overlap in phenotypic gene expression with related cell types (i.e. notochordal cells and osteoblasts). The GRN for vertebrate chondrogenesis presented here serves as an initial synthesis of the molecular data available and highlights a number of potentially important transcription factors that deserve further attention from the scientific community. It is hoped that this presentation format will serve to assist in the design of further microarray studies aimed at clarifying, and verifying, the genetic interactions underlying cartilage formation, as well as serving as a platform for comparative studies in non-vertebrate lineages with the goal of uncovering the evolutionary origin of this complex and important tissue type.

Furthermore, invertebrate cartilages, particularly those found within cephalopods, may represent novel animal models for the study of cartilage from the prospective of evolution at the level of gene regulatory networks and molecular fingerprints. The presence of cartilaginous tissues in invertebrates opens new avenues for the exploration of biomechanical properties of cartilage and have the capacity to enlighten us on mechanisms of cartilage regeneration, two areas that have so far gone unexplored.

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Editor’s Note: Since there were no questions from the reviewers, there is no “Discussion with Reviewers” section.