Shifts in Gene Expression during Postnatal Chondroblast Differentiation *in vitro*

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INTRODUCTION

Treatment of cartilage defects is hindered by the limited capacity of that tissue for self-repair and healing. Better methods to stimulate cartilage tissue repair and growth are needed. Our limited knowledge of the molecular mechanisms involved in postnatal chondroblast differentiation (chondroinduction) confounds the design of novel repair strategies. Possible approaches to this problem are to stimulate cartilage growth and/or development, or to engineer replacement tissue. Both of these approaches would benefit from a clearer understanding of the molecular mechanisms that regulate postnatal chondroblast differentiation. As a first step toward identifying these mechanisms, we analyzed gene expression shifts in an *in vitro* model of chondroinduction. Our hypothesis was that cellular interactions with demineralized bone, which induces endochondral bone formation *in vivo*, would induce specific shifts in gene expression that determine the chondroblast phenotype.

CHONDROGENESIS DURING EMBRYONIC DEVELOPMENT

Cartilage formation and growth occur at several stages during development of the embryonic skeleton.[1-3] Chondroblasts first arise by differentiation of mesenchymal cells packed within skeletal condensations. These chondroblasts secrete a cartilage scaffold (anlage) of the skeletal element. The chondrocytes undergo hypertrophy and the cartilage matrix is calcified, resorbed, and replaced by bone. The formation of secondary ossification centers establishes growth plates in the epiphyses. Chondroblasts within the growth plate echo the programmed proliferation, hypertrophy, and cell death that occurred earlier in the skeletal condensations. In contrast, chondroblasts within articular cartilages proliferate as the limb grows. These cells remain metabolically active but are mitotically quiescent once skeletal maturity is reached. The molecular mechanisms that mediate each stage of embryonic chondrogenesis may have relevance for cartilage repair strategies.

Decades of work in chick, mouse, and other models have delineated several functional classes of genes that mediate aspects of embryonic development. A "master gene" paradigm has emerged for embryonic cell differentiation, in which induction of a specific DNA binding protein alters gene expression to bring about the differentiated phenotype. The prototype system is the mechanism of action of the basic helix-loop-helix protein, myoD, during differentiation of muscle cells. The "master gene" paradigm is emerging for other tissues of mesenchymal origin: the runt-domain protein Osf2/Cbfa-1 is essential for osteoblast development, and the nuclear hormone receptor PPARγ is required for development of adipose cells. Accordingly, there has been a search for a chondrogenic "master gene". There are several candidate genes, including the HMG-box protein, *Sox9*; the bHLH protein, scleraxis; and the homeobox protein, Hoxc8.

Peptide growth factors are another functional class of genes that influence embryonic chondrogenesis. TGF-β and its family members GDF-5 and the BMPs are essential for prenatal chondrogenesis. Several members of the Wnt family of secreted glycoproteins have been shown affect embryonic chondrogenesis.

The extracellular matrix can also influence chondrocyte differentiation and chondrogenesis during embryonic development. Null mutations in the mouse *col2a1* or *colXa1* genes result in abnormal cartilage elements. Mice carrying a null mutation for perlecan, a heparin sulfate proteoglycan, have defective growth plates and disorganized cartilage matrix.

A comparison of chondrogenic mechanisms in avian and mammalian development shows that there are some species-specific differences. For example, a loss-of-function mutation in the mouse *wnt5a* gene generates pronounced skeletal defects: there is shortening of the limb, and digits fail to form. These observations support an instructive role for Wnt5a in specifying chondroblast differentiation in mammalian limb development. In the embryonic chick limb, however, overexpression of Wnt5a retards growth by delaying or inhibiting maturation of prehypertrophic chondrocytes. This suggests that, in the chick, Wnt5a is a suppresser of chondrogenesis.

How do the mechanisms of prenatal chondrogenesis compare to those of postnatal chondrogenesis? It has been difficult to address this question experimentally because, in a normal individual, postnatal chondrogenesis does not occur

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Table I. Evidence for Chondroinduction of Human Dermal Fibroblasts by 7 Days in Culture with Demineralized Bone Powder

Histological[5]	Immunohistological[5,7]	Immunochemical[5]	Molecular[6,8]
Round cells in lacunae	Chondroitin 4-sulfate	Chondroitin 4-sulfate	Collagen II
Metachromatic ECM	Collagen II	Chondroitin 6-sulfate	Aggrecan link protein
		Keratan-sulfate	IGF-I

outside of the growth plate or the soft callus of fracture repair. If postnatal chondrogenesis is affected by genes other than those associated with prenatal chondrogenesis, opportunities for therapeutic intervention may be greater than originally thought.

IN VITRO MODEL OF POSTNATAL CHONDROINDUCTION

In vivo, postnatal chondroblast differentiation can be induced by demineralized bone powder (DBP). Animal studies show that, in response to subcutaneous implants of DBP, cells are attracted to the DBP, are converted to chondroblasts, and produce a cartilage matrix that becomes vascularized, resorbed, and replaced by bone and marrow. My collaborators, Drs. Shuichi Mizuno and Julie Glowacki, developed a novel *in vitro* model of chondroinduction by DBP.[4,5] A three-dimensional collagen sponge culture device was designed to mimic the geometry and density of DBP in *in vivo* implants. Human dermal fibroblasts (hDFs) were used as target cells for chondroinduction. When hDFs were cultured in DBP/collagen sponges for one week, they developed a chondroblast phenotype. Histologic, biochemical, and gene expression analyses were used to characterize the cartilage-like matrix that accumulated around hDFs in contact with DBP.[5-7] Those results established that chondroinduction by DBP could be accomplished *in vitro* (Table I). We could then begin to elucidate molecular and cellular events that mediate postnatal chondroinduction and chondrogenesis.

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN CHONDROINDUCED FIBROBLASTS

As a first step toward identifying mechanisms of postnatal chondroblast differentiation, we analyzed shifts in gene expression that occur in hDFs cultured in DBP/collagen sponges. To determine an appropriate timepoint for gene expression analysis, we evaluated evidence of chondroblast differentiation in DBP/collagen sponges cultured for 3 and 7 days. After 3 days, hDFs were distributed throughout the sponges and were attached to the collagen lattice; those cells that had migrated into the packet of DBP were attached to, and between the particles of DBP. Metachromatic extracellular matrix was not observed in the DBP/collagen sponges at 3 days. After 7 days, however, metachromatic extracellular matrix and cartilage proteoglycans were present.[9] Moreover, expression of cartilage-specific genes was not detectable in hDFs cultured in DBP/collagen sponges for 3 days, but was induced by 7 days (Figure 1). Three days was therefore taken to represent a timepoint at which early interactions were occurring between the cells and DBP, prior to expression of the chondroblast phenotype.

We used representational difference analysis, a PCR-based

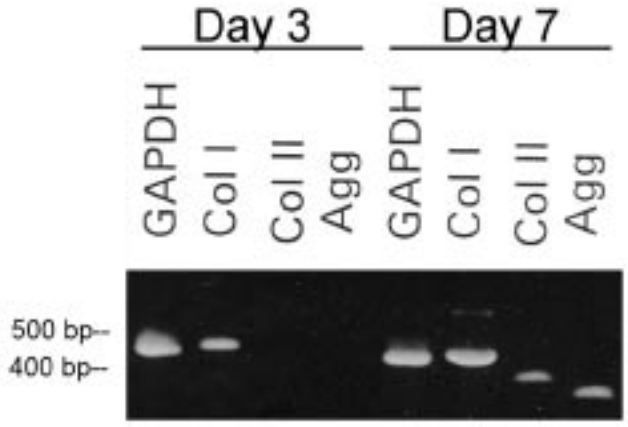

Figure 1. Gene expression in chondroinduced fibroblasts. RT-PCR was used to survey expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH, a "housekeeping" gene), type I collagen (Col I), type II collagen (Col II) and aggrecan (Agg) in human dermal fibroblasts (hDFs) chondroinduced for 3 and 7 days.

method of subtractive hybridization, to isolate a pool of genes whose expression was increased (Upregulated) in chondroinduced hDFs three days after exposure to DBP.[9] Several of the genes we identified were novel. Others corresponded to mRNA transcripts of unknown cellular function. Many Upregulated genes, however, corresponded to genes with known functions, including protein synthesis, cytoskeletal elements, extracellular matrix, and the regulation of gene expression.

Several aspects of each cellular process were represented among the functional classes of Upregulated genes. For example, the synthesis, aminoacylation, and export of tRNA from the nucleus are all required steps in protein synthesis. Different types of cytoskeletal elements were also identified: gene products associated with the actin- or microtubule-based cytoskeleton, gene products involved in cell adhesion, and gene products that function in messenger RNA attachment to the cytoskeleton. Genes involved in the regulation of gene expression fell into two classes: transcription factors and DNA helicases. Transcription factors provide gene-specific control of expression. In contrast, DNA helicases unwind strands of chromatin, thereby enabling access of transcription factors to their DNA sequence-specific binding sites. Helicases can also prevent transcription by re-winding chromatin.

SHIFTS IN GENE EXPRESSION IN CHONDROINDUCED FIBROBLASTS

The pool of genes identified as Upregulated in chondroinduced hDFs on day 3 represents a "molecular snapshot" of the DBP-induced changes in gene expression at that time. To better understand gene expression shifts during chondroinduction and chondrogenesis, we characterized the kinetics of

expression of Upregulated genes in chondroinduced hDFs. We found two patterns of expression for genes that were identified as Upregulated on day 3. In the "transient" pattern, gene expression levels were elevated on day 3 and declined thereafter. In the "intermediate" pattern, the increase in gene expression was sustained for 7 days or longer. Those patterns, transient and intermediate, were distinct from expression of abundant cartilage-specific extracellular matrix genes, which were not upregulated at day 3 and showed a later increase.[9]

The observed gene expression patterns, together with histologic and biochemical evidence of the chondroblast phenotype, suggest that there are specific stages of induced chondrocyte differentiation in our experimental system (Figure 2). Three days after exposure to DBP, the cell is between induction and overt differentiation. Gene expression shifts are already detectable in this early, determined neochondroblast stage. Elevated expression of some genes is transient. Elevated expression of other genes continues while cartilage-specific matrix is being actively synthesized in the differentiated neochondroblast stage. Cartilage-specific matrix gene expression, which is present at the differentiated neochondroblast stage, continues to increase until the cell reaches the mature stage of a chondrocyte.

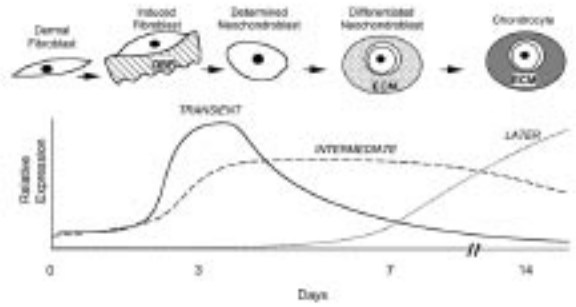

Figure 2. Patterns of gene expression correspond to cellular stages of postnatal chondroinduction *in vitro*. DBP, demineralized bone powder; ECM, extracellular matrix.

Table II. Expression in Cartilage of Genes Identified by Representational Difference Analysis as Upregulated in Chondroinduced Human Dermal Fibroblasts.

Functional Class	Documented In Cartilage	Not Documented In Cartilage
Extracellular	Annexin A5	
	COL3A1	
	COL5A2	
	COL6A3	
	COL11A1	
	Laminin	
	Thrombospondin-1	
Cell adhesion, cytoskeletal	α-11 integrin	Erythroblast macrophage protein
	β-1 integrin	Filamin
		RhoGAP1
Post-translational enzymes	Lysyl hydroxylase 2	Aspartyl β-hydroxylase
	Lysyl oxidase	Lysyl oxidase-like protein 1
Growth factors	FGF-2	CLF-1
	IGF-BP3	PDGF-C

CONCLUSIONS

In summary, we have identified pools of genes whose expression is increased in postnatal fibroblasts chondroinduced by DBP. Our data indicate that specific gene shifts in functional families (Table II) occur prior to full expression of cartilage-specific matrix genes and synthesis of extracellular matrix. Numerous cell processes are affected, including protein synthesis, cytoskeletal elements, extracellular matrix, and the regulation of gene expression. An increase in protein synthesis and export is expected in cells undergoing chondroblastic differentiation. Changes in cytoskeletal and cell polarity genes are consistent with the differences in cell shape of fibroblasts (spindle-shaped) and chondrocytes (round). Upregulation of helicases implies that changes in chromatin structure may occur to permit silencing of some genes (fibroblast-specific) and expression of others (chondroblast-specific). Overall, the diversity of cellular processes affected by DBP suggests a major reorganization of cell shape and function that occurs early in chondroblast differentiation. This information represents the first clues as to how postnatal chondrogenesis is accomplished.

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