Transcriptional Regulation of Chondrocyte Differentiation

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INTRODUCTION

Chondrocyte differentiation during embryonic development is a highly regulated process. Coordinated actions of extracellular matrix components, peptide factors, cell shape changes and intercellular communications result in the expression of "master genes" - transcription factors – that control expression of the chondrocyte phenotype. The mechanisms that regulate chondrocyte differentiation post-natally, however, are less well understood. One project in our lab is to identify those mechanisms, in particular the transcription factors that regulate chondroinduction of post-natal fibroblasts by demineralized bone powder (DBP). The objectives are to define the transcriptional networks that are activated in post-natal fibroblasts by DBP, and to make comparisons with regulation of chondrocyte differentiation during embryonic development.

TRANSCRIPTIONAL REGULATION OF CHONDROCYTE DIFFERENTIATION DURING EMBRYONIC DEVELOPMENT

Chondrocyte differentiation during embryonic development consists of a series of highly regulated events. Cells that are loosely packed in the mesenchyme undergo condensation into tightly organized aggregates. This process is mediated by communications between cells (via gap junctions) and with the matrix (via adhesion molecules), as well cytoskeletal-linked changes in cell shape.

After condensation is complete, chondrogenic differentiation ensues and expression of cartilage-specific proteins is initiated. Differentiation entails a switch from production of an extracellular matrix containing collagens I and III to production of the cartilage-specific collagens II, IX, and XI, and also aggrecan. The chondrocytes become flattened and undergo unidirectional proliferation consisting of a population of centrally localized type II collagen-expressing chondrocytes and more peripherally localized type I collagen-expressing perichondrial cells. The next stage is prehypertrophic chondrocytes—cells making the transition from proliferating chondrocytes to hypertrophic chondrocytes. Prehypertrophic chondrocytes form a specialized extracellular matrix that contains type II collagen. The cells then transit to mature hypertrophic chondrocytes that express type X collagen.

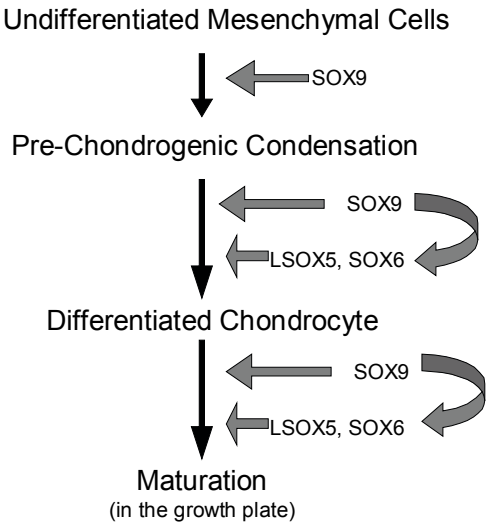

Figure 1. Positive regulation of chondrocyte differentiation during embryonic development by SOX family transcription factors.

Role of SOX family transcription factors.

SOX9 is an essential transcription factor that acts at several stages of chondrogenesis (Figure 1). During mouse embryonic development, SOX9 is expressed at high levels in all chondrogenic precursor cells and in chondrocytes throughout the deposition of cartilage-specific matrix (1, 2). Cartilage genes Col2a1 and Col11a2, as well as CD-RAP and AGGRECAN, have been identified as direct targets for SOX9 during chondrogenesis (3) Bridgewater (4-6). Mouse SOX9-null embryonic stem cells are unable to undergo mesenchymal condensation and thus cannot proceed through differentiation to express the chondrocyte-specific markers Col2a1, Col11a2, and AGGRECAN (7). In the absence of SOX9, there is a complete block at the stage of mesenchymal condensation, resulting in a complete absence of chondrocytes. Conditional ablation of the SOX9 gene after mesenchymal condensation has taken place results in a severe generalized chondrodysplasia (8). Therefore, SOX9 appears to be required both before and after the condensation stage of chondrocyte development.

Two other members of the Sox family, L-SOX5 and SOX6 also play a vital role in chondrocyte differentiation. L-SOX5 and SOX6 are coexpressed with SOX9 in chondroprogenitors and differentiated chondrocytes (9). L-SOX5 and SOX6 cooperate with SOX9 to activate the Col2a1 enhancer and gene expres-

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sion (9). Mice carrying mutations in both L-SOX5 and SOX6 develop a severe, generalized chondrodysplasia characterized by absence of cartilage, and the presumptive chondrogenic cells are largely arrested at the stage of mesenchymal condensation (10). Inactivation of SOX9 in limb buds before mesenchymal condensation abolishes expression of SOX5 and SOX6, indicating that SOX9 is required for expression of SOX5 and SOX6 (8).

Mutations in SOX that affect human skeletal development.

Expression of SOX9 is critical for normal cartilage formation in humans. Mutations in the human SOX9 gene are associated with the development of campomelic dysplasia, a human dwarfism condition that affects all cartilage-derived structures and can result in bowing of femora and tibiae, hypoplastic scapulae, a missing pair of ribs, lack of mineralization of thoracic pedicles, pelvic malformations and bilateral clubfeet (11, 12). De novo heterozygous loss-of-function mutations in the SOX9 gene have been identified in many campomelic dysplasia patients (13, 14), identifying haploinsufficiency for SOX9 as the cause for the phenotype and assigning a key regulatory role for SOX9 in chondrogenesis.

More than 20 amino acid substitutions have been identified in the 79 amino acid DNA-binding motif HMG domain of SOX9 that result in CD (15). Recent studies have also identified and characterized *de novo SOX9* mutations located within in a dimerization domain of the protein (12). Those mutations interfere with promoter activation that requires binding of SOX9 dimers. These studies support a functional role for SOX9 in campomelic dysplasia.

TRANSCRIPTIONAL REGULATION DURING POST-NATAL CHONDROINDUCTION

Our collaborators in the Department, Drs. Julie Glowacki and Shuichi Mizuno, have developed an *in vitro* culture system for chondroinduction of post-natal cells that models an early stage of the endochondral ossification that is induced *in vivo* by demineralized bone powder (DBP). In that system, human dermal fibroblasts (hDFs) are cultured in porous, 3-dimensional collagen sponges filled with DBP (16). After 7 days, the chondroinduced hDFs express cartilage-specific matrix collagens and proteoglycans (17). Induction of cartilage genes is preceded by altered expression of several functional classes of genes, including other matrix components, cytoskeletal elements, secreted peptide factors, and apparatus for protein synthesis (18). The global shifts in gene expression suggested that transcriptional regulators were affected in hDFs exposed to DBP. We have used two approaches to define the transcriptional regulators and networks employed during chondroinduction of hDFs.

Gene expression analyses.

Comparisons of gene expression revealed two types of transcriptional regulators that are affected in hDFs exposed to DBP: transcription factors (such as ID-2, ID-3, and TRAX) (18, 19) and chromatin modifying enzymes (such as helicases and histone deacetylases) (18). Transcription factors (TFs) bind to regulatory sequences located within in a gene. They enhance (or inhibit) expression of that gene by interacting with the nuclear transcriptional apparatus. In contrast, chromatin-modifying proteins can either "unwind" the DNA double helix to make a particular gene more accessible to transcription factors, or act to wrap the DNA tightly around histones and limit TF access. Altered expression of chromatin modifying proteins suggests that there is silencing of fibroblast genes concomitant with the induction of cartilage-specific genes in hDFs exposed to DBP. The TFs that were identified as altered by DBP in our gene expression analyses are not known to regulate embryonic chondrocyte differentiation. We therefore examined expression of candidate genes directly, by quantitative PCR. Results to date indicate that transcriptional regulators of embryonic chondrocyte differentiation are activated during post-natal chondroinduction in this system.

Array-based analysis of transcription factor activity.

The large number of genes that show altered expression prior to the chondroblast phenotype suggest that many transcriptional networks were affected by hDFs' exposure to DBP. A serial analysis of potential TFs was impractical, so we chose to screen many TFs simultaneously. cDNA arrays have been useful to identify changes in expressed genes during post-natal chondrocyte differentiation (19). TF activity can be altered, however, by post-translational protein modifications that cannot be detected via gene expression. Therefore, we used a method that would be sensitive to changes in abundance and/or activity to identify changes in transcriptional regulators in hDFs chondroinduced with DBP.

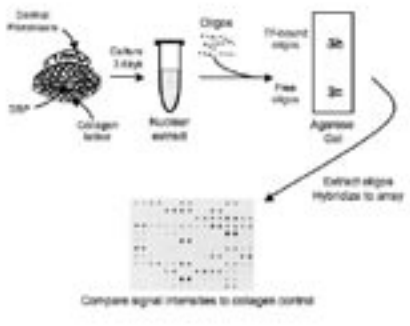

Figure 2.
Overview of array-based assay to measure transcription factor activity.

DNA binding of TFs was measured *in vitro* as a surrogate for their activity (TranSignal DNA/Protein Arrays, Panomics, Inc) (Figure 2). Nuclei were isolated from hDFs cultured in DBP/collagen and control collagen sponges for 3 days. The nuclear proteins were extracted and were incubated with oligonucleotides that encode DNA binding sites for specific TFs. Gel electrophoresis was used to isolate TF-bound oligos. The purified oligos were treated to dissociate the proteins and were hybridized to nylon arrays of complementary DNA sequences. A positive signal on the array, therefore, indicated that the corresponding TF was active. The signal intensity was measured and used to evaluate TF activity in hDFs exposed to DBP and collagen sponge controls. TF activity was then evaluated by measuring the signal intensity of the probes bound to the array.

Human dermal fibroblasts exposed to DBP for 3 days showed altered activity of many TFs (20). Of note, many transcription factors whose expression are regulated during development, but are not known to play roles in skeletal tissues, were affected. That result suggested that exposure to DBP activates a differentiation program in hDFs. In addition, many transcription factors that are activated via cell-surface receptors' interactions with ligand were also affected in hDFs cultured with DBP. That result suggested that a function of DBP-induced peptide factors and matrix components is to provide additional instructions to hDFs during chondroblast differentiation. This "feedback loop" may be necessary to support the differentiation process, given the absence of a supportive matrix environment, such as is present during embryonic development.

DISCUSSION

Surveys of transcription factor expression activity in a model of post-natal chondroinduction revealed similarities and disparities with embryonic chondrocyte differentiation. In each case, matrix components and secreted peptides/growth factors provide instructions and influence gene expression. Those extracellular signals work in concert with developmentally-regulated transcription factors to coordinate a differentiation program. The specific players, however, differ in pre-and post-natal cells.

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