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REGULATION OF CHONDROCYTE GENE EXPRESSION

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1. ABSTRACT

Extracellular influences known to affect the regulation of chondrocyte biosynthetic and catabolic activity have been shown to include soluble factors, extracellular matrix and mechanical stress. A balance of these numerous extracellular influences is required for normal function of articular cartilage. It is likely that OA is the result of an imbalance of regulatory influences, ultimately resulting in deleterious changes in gene expression, altered extracellular matrix (ECM) and tissue degeneration.

Molecular signalling via soluble mediators has been shown to be crucial to cartilage homeostasis. A number of vitamins, hormones, growth/differentiation factors and cytokines have been implicated in chondrocyte differentiation and cartilage metabolism. During normal maintenance, as well as in aging and pathology, these soluble factors can significantly influence the physical properties and the function of cartilage.

Chondrocytes, like cells in other tissues, exist within an information-rich extracellular environment consisting of ECM molecules, a milieu which interacts with and modulates the activity of growth factors, hormones and ECM remodeling enzymes. Cell surface matrix receptors, including a family of proteins known as integrins, connect

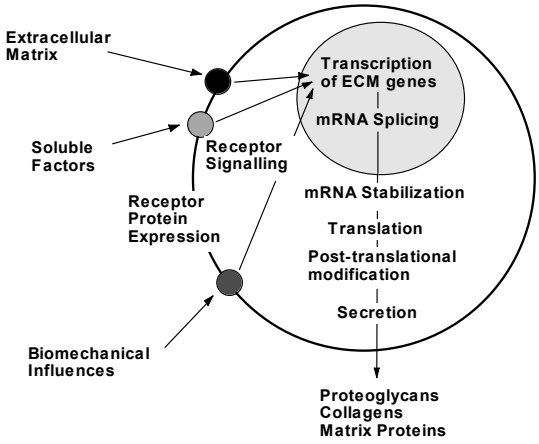

Figure 1. Extracellular influences upon chondrocyte gene expression. Receptor signaling influences ECM gene transcription, and additional regulatory events may occur at multiple steps preceding secretion.

structural information in the ECM to a complex cellular response mechanism in the cell's interior. Integrins on cell surfaces detect and transduce signals in a cooperative manner with other adhesion receptor classes and/or growth factor receptors.

The effects of mechanical stress upon a number of chondrocyte biological parameters has been examined in several laboratories. Other investigations have addressed the mechanism by which mechanical force affects biochemical and biosynthetic processes in chondrocytes, in particular synthesis of aggrecan, a major component of the cartilage ECM.

Each of these extracellular influences upon chondrocyte metabolism may affect regulation of chondrocyte ECM biosynthesis at many levels, including mRNA transcription, RNA splicing, nuclear transport, protein translation, post-translational modification, intracellular vesicular transport, and protein secretion. Transcriptional regulation of some of the major protein and proteoglycan components of the cartilage ECM has been examined in a number of species, and promoters have been characterized for aggrecan, link protein and collagen type II genes. There is evidence that gene expression may be altered in OA cartilage, providing clues as to which subsets of genes expressed in chondrocytes may be considered relevant to OA pathophysiology.

2. INTRODUCTION

Cartilage is not a distinct and homogeneous tissue, and the chondrocyte is not a discrete cell. Elastic cartilage and fibrocartilage are obvious examples of this principle, and hyaline cartilages in different anatomical locations are unique and highly specialized tissues. Chondrocytes within these hyaline cartilages exhibit regional phenotypic specialization. To make matters even more complex, chondrocyte phenotypic expression is dependent upon the developmental age of the tissue or maturational stage during growth. Changes occur in chondrocyte gene expression that are characteristic of aging and pathology.

The following review will examine current thinking with respect to regulation of extracellular matrix (ECM) gene expression in chondrocytes. For the purposes of this review, "regulation" is broadly defined as "influencing cellular activity" and "gene expression" can be regulated at many levels beginning with gene transcription and culminating in secretion. Certain general principles will be discussed concerning regulatory influences upon these cells (figure 1). Extracellular factors can alter patterns of transcription factor expression, affecting the expression of a subset of the genome transcribed in cells we identify as chondrocytes. Following gene transcription, additional levels of regulation must be considered, each of which can significantly affect what we consider to be the overall "phenotype". Alternative splicing events are known to occur for many chondrocyte-specific gene products. The persistence of gene expression is dependent upon the rate of transcription and the stability of the mRNA, and other factors. Additional regulation may occur at the levels of translation of the mRNA, post-translational modification, and secretion of the completely processed gene product. Perhaps by understanding the effects of these extracellular influences on the expression of individual genes, we will eventually arrive at an understanding of more global alterations occurring in cartilage at the level of the tissue, as well as at the level of the joint as an organ, during development, maturation, aging and pathology.

3.EXTRACELLULAR REGULATORY INFLUENCES ON CARTILAGE MATRIX GENE EXPRESSION

3.1. Soluble factors

Molecular signaling events via soluble mediators have been shown to be crucial to the process of skeletogenesis, cartilage homeostasis, aging and pathology. A number of vitamins, hormones, growth/differentiation factors and cytokines have been implicated in chondrocyte differentiation (also reviewed in (1)). During normal maintenance of cartilage in mature animals, regulation of chondrocyte matrix metabolism by soluble factors can significantly influence the physical properties and the function of cartilage (2). Some of the better characterized soluble factors will be discussed below.

3.1.1.Vitamins

3.1.1.1 Retinoic acid

The vitamin A derivative retinoic acid (RA) is a well characterized soluble mediator of cartilage development. During the early stages of chondrocyte differentiation *in vitro*, retinoic acid has been found to induce dedifferentiation of chondrocytes and acts as an inhibitor of chondrogenesis. RA treatment of differentiated chondrocytes in culture results in reversion to a fibroblastic phenotype, as indicated by a cessation of type II and IX collagen synthesis, and onset of type I and III collagen

production (3-8). Retinoic acid has been shown to influence gene expression directly, with a secondary change in cell shape (9). Proteins characteristic of the dedifferentiated phenotype were produced by RA-treated chondocytes suspended in methylcellulose, with no change from a rounded morphology. In these experiments, a single, relatively high dose of RA was employed. Other results suggested that cell-ECM interactions played a major role in RA-induced phenotypic modulation. Sanchez *et al* (10) reported that RA treatment did not significantly effect expression of ECM genes in quail chondrocytes if the initial morphological alteration from spherical floating chondrocytes to adherent chondrocytes was blocked. Together, these results suggest that actin organization rather than cell shape changes may be critical in modulation of the chondrocyte phenotype.

The importance of RA dosage became evident in experiments with serum-free cultures of chick limb bud mesenchymal cells (11) and chick craniofacial mesenchyme (12). In contrast to the effects of RA noted above, concentrations of RA known to cause skeletal duplication *in vivo* were found to significantly enhance *in vitro* chondrogenesis. In these same systems, high RA concentrations inhibited chondrogenesis. Variability in induction and inhibition of chondrogenesis by RA is likely to be due to the concentration of RA in individual experiments.

The effect of exogenous RA appears to depend on the differentiation state of the chondrocyte. At later stages of chondrocyte differentiation, RA promotes hypertrophy and type X collagen production (13). Immature chondrocytes from chick sterna were allowed to differentiate in culture and were treated with 10-100 nM RA at different culture times. It was observed that more immature chondrocytes failed to express type X collagen in response to RA. RA did induce type X collagen in more developed chondrocytes (14). Developmentally-related responsiveness to RA was determined by analyzing chondrocytes isolated from the cephalic region of different-aged chick sterna. Treatment of mature chondrocytes with RA stimulated an increase in alkaline phosphatase activity and promoted expression of other mineralization-related genes (15). Parathyroid hormone, an agent known to modulate maturation and mineralization of growth plate chondrocytes could reverse this process.

RA is a powerful resorbing agent for articular cartilage. It has been shown to be effective at physiological doses (10^{-8} to 10^{-10} M) by enhancing catabolism and inhibiting proteoglycan synthesis (16).

A number of proteins are known to play a role in RA stimulation, but the protein(s) mediating RA effects in chondrocytes have not been identified. CRABP is expressed during limb bud formation in chick development (17), and may influence gene transcription by regulating the effective concentration of RA that reaches the nucleus (18). Levels of CRABP II mRNA are responsive to RA treatment of embryonic carcinoma (EC) cells (19). No expression of CRABP was seen in RA-treated or control chondrocytes, indicating that CRABP protein may not be involved in the RA-induced modulation of chondrocytes (20).

3.1.1.2. Vitamin D_3

Vitamin D has been shown in numerous studies to play a role in the maturation of epiphyseal chondrocytes. Animals deficient in vitamin D demonstrate marked enlargement of the epiphyseal growth plate, disorganization of chondrocyte cell columns and inhibited cartilage matrix calcification, all of which are reversible by direct vitamin D administration (21). Vitamin D may regulate calcification by regulating chondrocyte synthesis of proteoglycans. Horton *et al* (22) demonstrated that the active metabolite of vitamin D, $1,25(OH)_2D_3$ produced a concentration-dependent reduction in aggrecan synthesis in an immortalized rat chondrocyte cell line. The reduced expression of the aggrecan gene was due to decreased steady-state levels of aggrecan mRNA, which were the consequence not of decreased transcription, but of accelerated aggrecan mRNA turnover. The effects of vitamin D on aggrecan synthesis are variable depending upon the type of chondrocyte and culture conditions. For example, $1,25(OH)_2D_3$ was shown to stimulate proteoglycan synthesis by chondrocytes isolated from rabbit or human articular cartilage (23). Gerstenfeld *et al* (24) showed that chondrocytes derived from embryonic chicken caudal sterna, a tissue that remains permanantly hyaline cartilage *in vivo*, could assume a more hypertrophic phenotype when treated with $1,25(OH)_2D_3$, with the activation and suppression of specific genes associated with this phenotypic transition. This transition was accompanied by morphological changes which in and of themselves promoted secondary changes in gene expression.

Both vitamin D_3 and $1,25(OH)_2D_3$ are considered secosteroids, bearing a structural relationship to classical steroid hormones. Vitamin D receptors in the traditional view have belonged to only one class, which includes cytosolic/nuclear proteins that bind specifically to the secosteroid as it transits the plasma membrane. These proteins subsequently interact with gene promoters in the nucleus to upregulate or downregulate gene expression (25). A large number of studies related to rapid responses to $1,25(OH)_2D_3$ have suggested that not all of the effects of $1,25(OH)_2D_3$ can be explained by receptor-hormone complexes binding to nuclear sites. Recently, a membrane receptor specific for $1,25(OH)_2D_3$ has been found to be present on growth plate chondrocytes (26). This specific membrane receptor was shown to be, at least in part, responsible for rapid increases in protein kinase C (PKC) in response to $1,25(OH)_2D_3$.

3.1.1.3. Ascorbic acid

Stimulation of proliferation by ascorbic acid has been observed in cultures of rabbit chondrocytes plated at high density (27-29) rabbit cartilage explants (30,31), chick embryo chondrocytes (32) and bovine articular chondrocytes (33).

Ascorbate is required for *in vitro* differentiation of mouse embryo chondrocytes. Chondrocytes

enzymatically dissociated from mouse embryo tibae grow in monolayer culture with a fibroblast-like phenotype. Differentiation can be induced by growing the cells in anchorage-independent conditions with ascorbic acid supplementation (34). After treatment with ascorbic acid, cultured chondrocytes undergo changes in gene expression characteristic of hypertrophy, including expression of type X collagen and alkaline phosphatase. Evidence suggests that although this inductive effect may not be a direct stimulation of transcription, it is not secondary to the ascorbate-stimulated production of a collagen rich matrix (35).

Ascorbic acid has been shown not only to stimulate ECM formation by bovine chondrocytes in culture, but it can also alter their phenotypic expression (36). Ascorbic acid can influence chondrocyte collagen production by modulating steady-state procollagen mRNA levels (37), as well as through postranslational processing of procollagen (38). The effect of ascorbic acid on chondrocyte proteoglycan synthesis has also been studied. Conflicting results, however, have been obtained (27,28,32,39-44). A possible direct effect of ascorbate upon proteoglycan core protein synthesis was suggested by the finding of a 40% decrease in core protein synthesis in guinea pig scorbutic cartilage (45). Our laboratory provided the first direct evidence that proteoglycan synthesis may be regulated by ascorbic acid at the mRNA level (33). The presence or absence of ascorbate did not appear to influence the secretion of proteoglycans although collagen secretion was profoundly affected by ascorbate deficiency (46).

3.1.2. Hormones

3.1.2.1. Thyroid hormone

Thyroid hormone actions on cartilage and bone, and the interactions of T_3 with other hormone signalling pathways, has been recently reviewed by Williams et al. (47). Certain direct effects of T_3 upon chondrocyte gene expression have been observed. For example, specific nuclear binding sites for T_3 have been found in cultured human fetal epiphyseal chondrocytes, cells which respond to T_3 *in vitro* with increased alkaline phosphatase activity (48). In another study, rat epiphyseal chondrocytes were shown to respond to T_3 with upregulation of alkaline phosphatase activity and IGF-I receptor mRNA levels (49). In both studies, however, it was not possible to conclude that the observed responses were due to transcriptional activation. Numerous *in vitro* studies, however, have demonstrated that chondrocyte differentiation requires thyroid hormone (47). Genes responding directly to T_3 in these investigations, however, have not yet been elucidated.

3.1.2.2.Steroid hormones

Dexamethasone has been shown to promote proliferation of skeletal cell precursors. *In vitro*, mesenchymal cells derived from avian connective tissues can differentiate into muscle, fat, cartilage and bone in a time and concentration dependent manner (50). Direct and sex-specific effects of 17beta-estradiol and testosterone on costochondral differentiation and ECM protein production *in vitro* have been reported (51-53). In a subsequent study, receptors for 17 beta-estradiol and testosterone were demonstrated on rat costochondal chondrocytes (54), suggesting that these receptors may mediate direct regulation of metabolism by sex hormones. The sex-specific effect of 17beta-estradiol is thought to be due to differences in receptor number between male and females. The sex-specific effect of testosterone may be regulated at a post-receptor level, since no sex-specific differences in binding capacity were observed.

3.1.3.Growth and differentiation factors/Cytokines

Numerous soluble mediators implicated in induction of mesoderm and differentiation of the cartilaginous anlage during limb development, and in the growth and differentiation of chondrocytes within the growth plate have been previously reviewed (1,55). These include (among others) the transforming growth factor-beta superfamily (inclusive of TGF-beta and bone morphogenetic protein (BMP) subfamilies), fibroblast growth factor (FGF) family, insulin-like growth factor (IGF) and growth hormone (GH), chondromodulin I and II (ChM-1 and II), connective tissue growth factor (CTGF), interleukin-1 beta (IL-1beta), indian hedgehog and parathyroid hormone-related peptide (PTHrP).

3.1.3.1. TGF-beta superfamily proteins: Bone Morphogenetic Proteins (BMP) and Cartilage-Derived Morphogenetic Protein (CDMP)

Two novel members of the BMP family, designated Cartilage-derived Morphogenetic Proteins-1 and -2 have been described (56), which may be a novel BMP subfamily, based on the high similarity of their carboxyl-terminal domains (also reviewed in (57)). CDMP-1 may play an important role in the development of the appendicular skeleton. Transcripts for CDMP-1 were detected during human embryonic development in the precartilage mesenchymal condensations of the developing limb, and in cartilaginous cores of long bones, with no expression in the axial skeleton. The importance of CDMP-1 as a physiological chondrogenic signal was confirmed by the demonstration of a null mutation in Gdf-5 (the mouse homologue of human CDMP-1) as the mutation responsible for the brachypodism phenotype in mice. This mutation affects several steps in early limb development, and results in shortening of limbs (58).

3.1.3.2. Fibroblast growth factor (FGF) and fibroblast growth factor receptors (FGFr)

The fibroblast growth factor family members possess different degrees of homology, but similarly promote proliferation of cells of mesodermal and neuroectodermal origin. In particular, they exert major effects upon prechondrogenic mesenchymal cells as well as differentiated chondrocytes. The fibroblast growth factors and their receptors have been shown to play critical roles in bone growth. Aspects of FGF effects on chondocyte differentiation have been reviewed by Cancedda *et al.*(1). These proteins interact with ECM and, in particular, with heparan sulfate chains of cell-surface as well as ECM heparan sulfate proteoglycans. Signal transduction occurs following subsequent FGF binding to membrane receptors.

Mutations in FGF receptors, in particular FGFR3 can result in a spectrum of phenotypes ranging from hypochondroplasia (59), characterized by moderate disproportionate shortness, to thanatophoric dysplasia (60,61), characterized by very short limbs, an underdeveloped spine, and a small thorax that ultimately results in death through respiratory distress (reviewed in (55)).

3.1.3.3. Growth Hormone GH) and Insulin-like Growth Factors (IGF)

Growth hormone, in association with insulin-like growth factors (IGF I and II) has been shown to be an important regulator of longitudinal bone growth in mammals, most likely acting directly on chondrocytes of the epiphyseal growth plate (62), an action mediated by a membrane associated GH-receptor (GH-R) (63). The function of GH in regulation of avian longitudinal bone growth has not been as clearly defined. In recent studies, growth hormone has been shown to inhibit the differentiation (from proliferative to hypertrophic cells) of avian epiphyseal growth-plate chondrocytes (64). Treatment of proliferating chondrocytes with chicken GH caused an increase in collagen type II gene expression, and a decrease in the appearance of osteopontin in the medium. Ascorbic acid-stimulated increases in alkaline phosphatase activity were inhibited by GH as well. It was concluded that GH inhibited differentiation in growth plate chondrocytes, sustaining their proliferative state, while maintaining their sensitivity to other growth factors such as EGF.

In a subsequent study, it was demonstrated that the level of expression of the growth hormone receptor (GH-R) gene is independent of the state of chondrocyte differentiation, but that only undifferentiated chondrocytes could respond to the hormone (65). It was suggested that the reduction of the differentiated chondrocytes response to GH could be due to the differentiation-dependent loss of the extracellular domain of the GH-receptor, which results in a lack of functional receptors on the cell surface. In addition, chondrocyte differentiation was associated with the generation of GH binding protein (GHBP). These authors speculated that as growth plate chondrocytes differentiate from the proliferative to the hypertrophic state, they secrete GHBP, which might be sequestered in the hypertrophic zone ECM where it plays a role in the regulation of the cartilage to bone transition. Treatment of ovine costal chondrocytes with GH stimulates insulin-like growth factor binding protein-2 (IGFBP-2) production (66). This was demonstrated to be a cell-type specific effect, since BP-2 production was not affected by GH in fibroblast cell cultures. Although the physiological significance of production of IGFBPs in the growth plate remains to be elucidated, this result suggests that GH may affect IGF activity by regulating production of IGFBP.

Stage-specific transcription factors may be critical to hormonal or growth factor-induced stimulation of proliferation and differentiation. One such factor, the CCAAT/enhancer-binding protein-alpha (C/EBP alpha) was found to be expressed in the perichondrial ring, in the germinal layer of the growth plate and on the surface of the rat tibial articular cartilage (67). The growth hormone receptor was found to have a similar distribution. To further investigate this relationship, C/EBP alpha levels were determined to be reduced in rib cartilage following hypophysectomy. Since GH treatment did not counteract this effect, however, it appears that pituitary hormones other than GH may regulate C/EBP alpha mRNA levels in growth plate.

There is evidence that the synthesis of hyaluronan by hypertrophic chondrocytes may be a principal factor in the interstitial expansion of the growth plate (68). This process appears to be regulated by factors which promote chondrocyte maturation. Growth hormone-deficient mice were found to possess smaller hypertrophic lacunae. Insulin-like growth factor treatment of growth plate cartilage in culture was demonstrated to stimulate the production of hyaluronan and resulted in the enlargement of lacunae (69).

3.1.3.4. Chondromodulin I and II (ChM I and II)

Proteins isolated from cartilage have been found to synergistically stimulate both growth of chondrocytes and proteoglycan synthesis. Two distinct factors have been described (70), which differ in terms of their affinity for heparin, one eluting from a heparin-Sepharose column with buffer containing 1.2 M NaCl (Chondromodulin I) and the other having a weaker affinity, eluting at 0.5 M NaCl (Chondromodulin II). ChM-I is a 25 kDa glycoprotein expressed specifically in cartilage which stimulates chondrocyte proliferation and proteoglycan synthesis. Chm-I was observed to stimulate colony formation of rabbit growth plate chondrocytes in agarose culture, and acted synergistically with FGF-2 in this system. Neame *et al.* (71) have estimated the content of ChM-I in cartilage to be >1mg/g tissue, which is consistent with requirements for a high dose (>200 ng/ml) to induce colony formation *in vitro*. Recently, Northern blot analysis has revealed that ChM-I mRNA was expressed in a regulated and tissue-specific manner in cartilage (72). The level of ChM-I mRNA was markedly changed in response to growth and differential stimuli in primary cultured chondrocytes. Chm-I mRNA was downregulated when the metabolic state was shifted from maturing to proliferating by treatment with FGF-2 and TGF-beta. Similarly, Chm-I mRNA was downregulated by negative regulation of differentiation by PTH-rP. Treatment with BMP-2, however, stimulated collagen type II synthesis without upregulating Chm I mRNA levels. Overall, these results suggest that ChM-I expression may be regulated in a manner dependent upon the differentiated state of the chondrocyte.

3.1.3.5. Connective Tissue Growth Factor

Connective tissue growth factor has been shown to be highly expressed in a chondrosarcoma cell line and in cultured rabbit growth chondrocytes, and little expression was observed in non-cartilaginous tissues (73). Connective tissue growth factor is a member of the CCN gene family which includes *cef*10/*cyr*61, *ctgf*/*fisp*-12 and *nov* (74). The *cyr*61 gene has been found to be expressed in developing cartilaginous elements and placental tissues (75).

Connective tissue growth factor, however, appears to have a pattern of expression distinct from *cyr*61 suggesting that their functions may be distinct (73)

3.1.3.6. Interleukin-1beta (IL-1)

IL-1, originally described as a monocyte-macrophage secreted protein acting as an immune modulator is believed to play a major role in the inflammation and joint destruction characteristic of rheumatoid arthritis and OA (76). This cytokine suppresses cartilage-specific collagen (77,78) and proteoglycan (79-81) production in cartilage and primary chondrocyte cultures and in immortalized human chondrocyte cultures (82). The effects of IL-1 on the expression of the human type II collagen gene have been shown to be mediated primarily at the level of transcription (83). IL-1 has also been shown to upregulate the production on non-cartilage collagen types by chondrocytes (77,84). This cytokine may play a role in normal development, as it has been detected in the cartilage resorption zone during endochondral ossification of immature mouse bone (85).

3.1.3.7. Indian Hedgehog and PTHrP

The process of endochondal ossification commences with aggregation of undifferentiated mesenchyme. Cells within these condensations differentiate to chondrocytes, except for those at the periphery, which become perichondrium. Formation of the cartilaginous anlage of the skeletal element is accomplished by chondrocyte proliferation and ECM deposition. Ultimately, cells in the central region of the anlage stop proliferating and become hypertrophic. The accompanying changes in the ECM in this zone permit vascular invasion. Together with vascularization is the appearance of bone marrow cells and osteoblasts, which replace the original cartilage with mineralized bone matrix. The zone of hypertrophic cartilage and the ossified region broadens until the bone shaft is nearly completely mineralized except near the ends, where there remains cartilaginous growth plates containing proliferating and transitional hypertrophic chondrocytes. Premature ossification of growth plate cartilage requires continued proliferation of chondrocytes and precise control of the differentiation process leading to hypertrophy. It is the fine control of this process that determines the dimensions of skeletal elements at maturity.

Indian hedgehog (Ihh) and Parathyroid hormone-related protein (PTHrP) function in a feedback loop that regulates the rate of chondrocyte differentiation to balance growth and ossification of long bones. Hedgehog proteins comprise a family of conserved molecules that regulate embryonic pattern formation. In vertebrates three hedgehog genes have been characterized, including Sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh). Dhh functions as a spermatocyte survival factor in the testes, whereas Ihh and Shh have been shown to have similar biological properties and can stimulate expression of the same genes (86). Although both Ihh and Shh appear to have the same signalling capabilities, there are spatial and temporal differences in their expression in the embryo that account for distinct embryonic roles.

A negative feedback loop initiated by Ihh is mediated by the perichondrium. PTHrP is expressed mainly in the periarticular perichondrium of developing cartilaginous skeletal elements early in bone development. Its receptor (PTH/PTHrP receptor) is expressed in prehypertrophic cartilage. High levels of PTH/PTHrP receptor are expressed by prehypertrophic chondrocytes. Following committment to hypertrophy, Ihh is transiently expressed. The secreted Ihh acts on the Ptc/Gli-expressing cells in the perichondrium adjacent to the prehypertrophic zone as well as on the periarticular perichondrium, which induces the expression of PTHrP. PTHrP then signals back to chondrocytes expressing the PTH/PTHrP receptor to prevent nondifferentiated chondrocytes from becoming hypertrophic.

3.2. ECM

Chondrocytes, like cells in other tissues, exist within an information-rich extracellular environment consisting of ECM molecules, growth factors, hormones and ECM remodeling enzymes. Cell surface adhesion receptors connect structural information in the ECM to a complex cellular response mechanism in the cell interior. Integrins on cell surfaces detect and transduce signals in a cooperative manner with other adhesion receptor classes and/or growth factor receptors (87).

3.2.1. Chondrocyte gene expression is influenced by ECM

The chondrocyte phenotype in culture is influenced by cell shape, cytoskeletal organization and adhesive interactions with the ECM (88). These parameters may similarly influence chondrocyte gene expression within cartilage *in vivo*. Disruption of normal cell-matrix interactions by proteases in OA cartilage could modify cytoskeletal organization resulting in altered gene expression. In one recent study (89), the effect of ECM depletion upon proteoglycan synthesis was examined. Cultured bovine cartilage explants were depleted of proteoglycans using *Streptomyces* hyaluronidase, testicular hyaluronidase, or collagenase. Only collagenase treatment stimulated a significant increase in proteoglycan synthesis, suggesting that collagen and matrix organization was more important than proteoglycan levels in controlling chondrocyte proteoglycan synthesis. In agreement with this observation, we have demonstrated that treatment of high density chondrocyte cultures with pronase and collagenase stimulated 9-10 fold increases in aggrecan and link protein mRNA (33). These observed changes in gene expression were likely related to interactions between chondrocyte integrins and a collagenous component of the cartilage ECM.

Regulation of expression of ECM proteins and their corresponding receptors on cell surfaces is an important component of tissue differentiation, and tissue remodeling during repair processes. When considering the inability of cartilage to undergo effective repair, it should be appreciated that during limb development chondrogenesis begins in an ECM that differs significantly from normal cartilage, containing abundant fibronectin, type I (90) and/or type III collagen (91). Cells must be

capable of disrupting interactions with the ECM during cell division and repair in a regulated manner, a process likely to involve proteolysis of ligands or receptors (92). Although implicated in the pathogenesis of OA, proteases are also likely to play a prominent role in normal regulation of chondrocyte-matrix interactions. Thus, dysregulation of the interactions occurring between ECM receptors and ECM macromolecules may be fundamental to the etiology of OA. A potential mechanism for the modulation of chondrocyte gene expression in OA could involve disruption of cell-matrix interactions mediated by integrins.

The ECM protein tenascin-C , a molecule involved in the genesis and function of articular chondrocytes (93), has been shown to be present at increased levels in human OA cartilage. Tenascin-C/cytotactin/hexabrachion is a large ECM glycoprotein detectable in some embryonic tissues (94). Tenascin possesses structural features of fibronectin, but has, in fact, been shown to interfere with attachment and spreading of cells in culture (95). Immunohistochemical analysis of skeletal development in rodents revealed tenascin in condensing mesenchyme of the cartilage anlage, but not the surrounding mesenchyme (96). Tenascin was absent from mature bone matrix, but persisted on periosteal and endosteal surfaces. Tenascin was also found to be abundant in forming cartilage nodules in chick embryo wing bud cultures, and chondrogenesis was enhanced when these cultures were grown on a tenascin substrate. These results suggested that tenascin-C may be part of an *in vivo* mechanism for chondrocyte development at the epiphysis of long bones and the maintenance of functionality during postnatal life. Tenascin-C has been shown to interact with the cell surface heparan sulfate proteoglycan syndecan-1 (97), suggesting that it could modulate the interactions and responsiveness of articular chondrocytes to growth factors that normally interact with syndecan.

3.2.2. Chondrocyte integrins and ECM ligands

The integrin multigene family of transmembrane adhesion receptors mediates attachment of cells to ECM macromolecules (98). Chondrocyte-specific integrins have been characterized in both normal and OA human cartilage, as well as in other mammalian and avian cartilages. Using a comprehensive panel of integrin isoform-specific monoclonal antibodies, normal human articular chondrocytes were found to display large quantities of the alpha 1beta 1, alpha 5 beta 1 and alpha v beta 5 integrin heterodimers, and lesser amounts of alpha 3 beta 1 and alpha v beta 3 heterodimers. The alpha v subunit-containing integrins were observed to be more abundant on chondrocytes in the more superficial layers compared to deeper layers of cartilage (99). Another study (100) demonstrated the presence of the alpha 2, alpha 5, alpha 6, alpha v and beta 1 chains on freshly isolated human fetal chondrocytes.

In a study to compare the distribution of alpha and beta subunits of the integrin superfamily in normal and OA cartilage (101), it was concluded that chondrocytes in both normal and OA cartilage express alpha 1, alpha 5, alpha v, beta 1, beta 4, and beta 5 subunits. Chondrocytes in OA cartilage were found to additionally express alpha 2, alpha 4, and beta 2 subunits. In agreement with a previous study (99), the alpha v subunit was found to be expressed by more chondrocytes in the superficial zone than cells of the deeper zones of human cartilage (101). Correlations have been drawn between patterns of integrin expression and OA lesion severity. Beta 1 integrins were found to correlate inversely with the severity of anatomical lesions (102,103).

Studies have been performed to elucidate integrin-ligand relationships likely to occur in cartilage. Chondrocytes isolated from the cephalic region of sterna fom 14-day-old chick embryos were found to use beta 1 integrins and required either Mg^{2+} or Mn^{2+} for attachment to culture plates coated with type I collagen, type II collagen and fibronectin. At least 3 alpha subunits could be identified by western blot analysis and immunoprecipitation, including alpha 3, alpha 5. and a putative alpha 2 (104). The observation that collagen type II binding was Mg^{2+} -dependent and RGD-independent suggested that alpha 2 beta 1 was the most likely candidate for the type II collagen receptor. In the same study, alpha 5 beta 1 was identified as the putative fibronectin receptor in chick chondrocytes on the basis of its sensitivity to RGD peptides, divalent cation requirements, and its localization in adhesion plaques in cells plated on a fibronectin substrate. A recent study has confirmed that the interaction of the alpha 5 beta 1 integrin with fibronectin is necessary for adhesion, spreading, and proliferation of both chicken and rabbit chondrocytes (105).

3.2.3. Integrins and Signal Transduction

Binding of ECM ligands to integrins can induce cellular responses (106). Disruption of normal integrin-mediated cell-matrix interactions may similarly influence the expression of ECM genes, matrix assembly and/or deposition. Matrix assembly by cultured cells is inhibited in the presence of a monoclonal antibody that inhibits binding of alpha 5 beta 1 integrin to fibronectin (107). Transfected cells expressing elevated levels of the alpha 5 beta 1 fibronectin receptor also show an increased level of matrix depositon (108). Integrins can also recognize ECM degradation products (98), a finding that suggests a role in regulating gene expression during tissue repair following proteolytic matrix depletion. After binding to ECM, integrins bind to cytoskeletal elements and promote cytoskeletal reorganization. Cytoskeletal alterations in response to a specific integrin-ligand combination may result in the increased or decreased expression of genes. Different alpha-beta subunit combinations recognizing the same extracellular ligand may promote distinctly different cellular responses. In fibroblasts, cytoskeletal reorganization occurs following focal contact formation during attachment to fibronectin or vitronectin (109). The cytoplasmic domain of the integrin beta 1 subunit can bind two focal contact structural proteins, alpha-actinin and talin, and focal contact sites can be disassembled by alpha-actinin fragment microinjection into living cells (110). The alpha subunit cytoplasmic domain may function to regulate interactions between the beta subunit cytoplasmic tail and cytoplasmic binding sites (111-113).

There is evidence that signalling strategies used by integrins differ from those used by growth factor receptors and other receptor types with kinase or phosphatase activities in their cytoplasmic domains. Focal contact sites on fibroblasts contain a number of non-enzymatic proteins at low levels that may play a regulatory role in signal transduction, including paxillin, a substrate for tyrosine kinases and tensin, which possess an *src* homology 2 (SH2) domain. SH2 domains recognize tyrosine phosphatase sites on other proteins and could potentially link regulatory and structural proteins at focal contact sites (109). Additionally, focal contacts have been shown to contain enzymatic, cytoskeleton associated regulatory molecules forming an "intracellular activation complex" including protein tyrosine kinase pp125FAK (FAK, focal adhesion kinase) (114) and protein kinase C (115). Nitric oxide, a potential mediator of cartilage pathophysiology in arthritis, can inhibit the assembly of the intracellular activation complex and the subsequent upregulation of proteoglycan synthesis that occurs following ligation of alpha 5 beta 1 to fibronectin (116).

Signal transduction pathways activated by growth factors and ECM macromolecules converge at the level of control of gene transcription (117). As an example, terminal differentiation of mammary gland epithelium into alveolar structures and production of milk proteins requires both lactogenic hormones and basement membrane contact. The signal from the basement membrane is mediated by integrins, as antibodies against the beta 1 subunit family blocks beta-casein production. Responsiveness to ECM and lactogenic hormones is conveyed by a 161 bp enhancer element in the 5' regulatory region of the beta-casein gene. In these mammary epithelial cells, degradation of basement membrane by metalloproteinases triggers the programmed cell death that accompanies involution (118). In several other systems, a similar requirement for ECM signals to regulate terminal differentiation programs has been demonstrated (119-123). This observation is interesting in that there appears to be expression of type X collagen in OA chondrocytes (see below), normally a product of "terminally differentiated" chondrocytes. It is interesting to consider that this aberration may be the result of altered cell-matrix interactions in OA cartilage.

3.2.4. Integrins and the cytoskeleton

We have observed rapid upregulation of chondrocyte link protein, aggrecan and collagen type II mRNA upon enzymatic ECM depletion and resuspension of bovine chondrocytes. This result may be explained on the basis of a of lack of ECM receptor (integrin) occupancy by ligands and subsequent cytoskeletal reorganization (33). Cell shape is an important factor determining the chondrocyte phenotype (124-126). Cells that are spread and flattened have been shown to be phenotypically unstable and will eventually "dedifferentiate" (127). When chondrocytes are grown in suspension (128) or under conditions where their spherical shape is maintained (124) the normal phenotype persists. There is evidence that disruption of cytoskeletal elements serves as the link between changes in cell shape and gene expression. Takigawa *et al* (129) showed that cytochalasin B, a microfilament modifying agent, induced rabbit costal chondrocytes to change from polygonal to nearly spherical. Glycosaminoglycan synthesis was increased. When chondrocytes were treated with colchicine, a microtubule-disrupting agent, the chondrocytes became flattened and an accompanying inhibition of GAG synthesis was measured. Newman and Watt (130) used cytochalasin D to induce cell rounding and noted that [^{35}S]sulfate incorporation into proteoglycan was stimulated. Mallein-Gerin *et al* (131) have shown by *in-situ* hybridization and immunocytochemistry that changes in aggrecan and type II collagen expression were not directly correlated with chick embro chondrocyte changes in shape and have proposed that chondrocyte shape change does not necessarily have a causative effect on phenotypic expression. Benya *et al.* (132) have also demonstrated that phenotypic modulation does not absolutely require a change in cell shape. Modification of microfilaments, rather than their complete disruption, is sufficient to allow reexpression of the chondrocyte phenotype in RA-treated "dedifferentiated" chondrocytes.

3.3. Biomechanical influences

The effects of mechanical stress upon a number of chondrocyte biological parameters has been examined in several laboratories. Other investigations have addressed the mechanism by which mechanical force affects biochemical and biosynthetic processes in chondrocytes, in particular synthesis of aggrecan, a major component of the cartilage ECM.

3.3.1. Biomechanical stress and chondrocyte metabolism

Mechanical stress applied to chondrocyte cultures can stimulate proliferation (133) and affect ECM protein biosynthesis. Static compression has been shown to decrease proteoglycan and protein synthesis (134-136) while dynamic compression stimulates ECM protein synthesis (135,137,138). Biomechanical forces may be one of the major factors in pattern development of skeletal tissues. Static compressive forces have been shown to promote chondrogenesis in embryonic limb bud mesenchyme (139).

Mechanical loading of cartilage *in vitro* results in increased fluid pressure (hydrostatic pressure), fluid exudation, and cell deformation (140,141). There are accompanying changes in tissue pH, streaming potential and streaming currents(142,143). Transport limitations resulting from reduction in the average pore size of the compressed ECM have been considered (136,144,145). Changes have been noted in cell and nucleus structure in statically compressed cartilage, correlating with local changes in aggrecan synthesis (146).

Studies on the effects of stress on metabolic activities of chondrocytes have shown an upper limit of applied compressive stress around 1.0 Mpa. Above 0.5 Mpa static compression irreversible damage and impairment of chondrocyte metabolic actitivies occurs in vitro (147,148).

The ECM is the medium through which mechanical signals are transduced to chondrocytes. There

is evidence that the minimal system showing typical responses to mechanical loading appears to be the cell and it's immediate pericellular matrix (149). In this study, the effects of mechanical compression on the biosynthetic activity of chondrocytes and the requirement for ECM was examined. It was determined that for static compression, the chondrocyte response could be related to the presence or absence of ECM, suggesting that cell-matrix interactions and extracellular physicochemical effects may be more important than ECM-independent cell deformation and transport limitations. Similarly, for dynamic compression, fluid flow, streaming potentials and cell-ECM interactions appeared to be more important than the small increase in fluid pressure, transport or cell deformation.

Integrins may serve to detect and interpret these extracellular signals and transmit them to the nucleus. The adhesion of chondrocytes and chondrosarcoma cells to collagen has been shown to be mediated by the integins alpha 1 beta 1 and alpha 2 beta 1 (150). The alpha 2 beta 1 integrin also serves as a receptor for the cartilage matrix protein chondroadherin (151). The integrin subunit alpha 2 has been shown to be upregulated in chondrosarcoma cells during mechanical stress, while the expression of beta 1 was unchanged (150). Recent data suggests a role for the alpha 5 beta 1 integrin in transduction of mechanical stimuli in chondrocytes. Primary monolayer cultures of human chondrocytes were shown to exhibit an electrophysiological response following intermittent pressure-induced strain evidenced by membrane hyperpolarization (152). Wright *et al.* (153) further determined that the hyperpolarization response was reduced when GRGDSP peptide (an alpha 5 beta 1 ligand) was added to the medium prior to cyclical pressure-induced strain, and no effect was observed when control peptide GRADSP was used. Furthermore, antibodies directed against the alpha 5 and beta 1 integrin subunits reduced the hyperpolarization response.

3.3.2. Biomechanical stimulation of Aggrecan synthesis

Valhmu *et al* (154) investigated the effects of short and long-term load-controlled compresson on the levels of aggrecan mRNA. They found that compressive stress of 0.1 Mpa on bovine cartilage explants for 1 hr. produced a transient upregulation of aggrecan mRNA synthesis, but at longer times levels of aggrecan mRNA returned to baseline values. They observed a dose dependent response to increasing levels of compressive stress over a range of 0-0.25 Mpa for 1 hr. and no stimulation at 0.5 Mpa. Longer periods of compression (24 hr) for a range of stress levels resulted in no elevation of aggrecan mRNA. The stimulatory effect of short-term compression was blocked by Rp-cAMP and U-73122, suggesting that the transient stimulation involved activation of cAMP and phosphoinositol signalling pathways. The transient increase in aggrecan mRNA may confirm a previous study showing an increase in aggrecan mRNA levels and proteoglycan synthesis in response to intermittent hydrostatic pressure of 10 Mpa (155). The observation that compression of cartilage results in differential effects on biosynthetic pathways for aggrecan, link protein and hyaluronan (156) suggests that not all ECM components are regulated uniformly by compressive stress.

Although static compression for long periods of time did not stimulate aggrecan synthesis in mature cartilage, static compressive force has been demonstrated to stimulate aggrecan expression during chondrogenesis *in vitro*. In these experiments a static compressive force was applied to mouse embryonic limb bud mesenchyme in collagen gels, a system that mimics early chondrogenesis. Cartilaginous nodules were formed, accompanied by ECM protein deposition. Compressive force was found to accelerate the rate and extent of chondrogenic nodule formation. Aggrecan mRNA levels in compressed cultures was higher than controls at 5 and 10 days of culture under static compression (139).

4. TRANSCRIPTIONAL REGULATION OF CARTILAGE ECM GENES

Regulation of chondrocyte ECM biosynthesis occurs at many levels, including mRNA transcription, RNA splicing, nuclear transport, protein translation, post-translational modification, intracellular vesicular transport, and protein secretion. Elements of aggrecan, link protein and collagen type II gene regulation are among the most thoroughly studied to date.

4.1. Aggrecan Gene Expression

The structure of the rat aggrecan gene, and a preliminary characterization of its promoter was determined by Doege *et al.* (157). There is a minor promoter initiating transcription 68 bp 5' of the major promoter site. The rat aggrecan promoter lacks TATAA or CCAAT elements, but possesses several putative binding sites for transcription factors. Several SP1-binding sites are found in the vicinity of the transcription start site, as is commonly seen in promoters of this type. Promoters lacking the TATA signal have also been found to show multiple transcription start sites. The 120 bp of 5' flanking sequence characterized in this analysis was 72% G+C, and contained four potential AP-2 sites, two of which overlapped with potential SP1 sites. The first exon sequence was also found to be GC-rich with four overlapping potential AP-2 sites. A sequence was found with similarity to sequence within the rat type II collagen promoter, and occurring in the same relative position. This sequence is a potential binding site for Nf-kappa B, a protein known to interact with cytokines. Both the aggrecan and COL2A1 promoters were found to contain numerous clusters of SP1 sites. The link protein gene promoter contains sequences in common with the aggrecan promoter as well. This conserved region in the aggrecan gene has predicted binding sites for the AP-2 factor. When comparing rat collagen, aggrecan and link protein promoters, however, no regulatory sequences were found to be conserved in all three genes.

Similar to the rat aggrecan gene, the mouse aggrecan gene (158) contains no TATAA sequence, and is transcribed from multiple transcription start sites, which differ from those determined for the rat, although the gene

structure is similar. As has been found for the rat link protein and type II collagen genes, there is a high G +C content in the sequence upstream of the transcription start site. Two glucocorticoid receptor binding sequences and one SP-1 site were found in this region. Inverted repeat sequences, as well as sequences showing sequence identity to the rat type-II collagen and rat link protein promoters which may play a role in cartilage-specific gene expression were also found. Scleraxis is a member of the basic helix-loop-helix transcription factor family which could be considered a candidate regulator of cartilage-specific gene expression. Liu *et al.* (159) demonstrated that scleraxis enhanced activity of a reporter construct containing an 8 kb fragment of the mouse aggrecan gene which included both the promoter and first intron. Scleraxis was found to bind to a region in exon 1 designated AgE, which contains two adjacently positioned E-box sequences. E-boxes are *cis*-acting elements located in the promoter/enhancer regions of tissue-specific genes. The AgE motif is conserved, with several substitutions, within the 5'-UTR of bovine, human, rat and mouse aggrecan genes (160). The presence of this motif in the 5'-UTR may be related to the observation of Valhmu *et al.* (161) that the 5'-UTR could stimulate transcription from the aggrecan promoter.

Pirok *et al* (162) cloned the promoter region of the chicken aggrecan gene. As was seen in the rat aggrecan promoter, the chick 5' flanking sequence lacked a classical TATA box, and contained multiple transcription start sites. The 5' flanking region of chick aggrecan contained three major transcription start sites, several putative *cis* elements and regions of potential secondary structure. Furthermore, it was demonstrated that the 1.8 kb region examined possessed tissue-specific promoter activity, and contained regions that produced activation or repression of reporter genes in two cell types in culture.

Numerous potential *cis* elements were found in the genomic fragment examined, but several were considered to be of considerable interest. Two copies of the sequence CACCTCC (CIIS2) were found. This sequence was suggested to represent a silencer motif in the COL2A1 promoter, and has been shown to inhibit type II collagen promoter transcription in fibroblasts. A second silencer consensus (CIISI) ACCCTCTCT was also found. A putative NF-I site may confer mesenchyme-specific regulation. Four regions contain the motif CACACA which may contribute to secondary structure (Z-DNA).

Valhmu *et al.* (161) characterized the promoter as well as transcriptional regulatory activities within the 5' and 3'-untranslated regions of the human aggrecan gene. The human aggrecan promoter includes several SP-1/AP-2 binding sites around the putative transcriptional start site, consistent with a suggested role for SP-1 in regulating initiation of trascription of genes with TATA-less promoters. A TATG repeat sequence was found at the predicted site for a TATA box. It was suggested that this might serve an equivalent role to the TATA box in promoters lacking the canonical sequence. Motifs were detected that might be related to modulation of aggrecan expression by mechanical forces, cytokines and other serum factors. These include STAT and NF-kappa B sites, which may be related to cytokine responsiveness, SSRE sites, which are potential regulators of shear stress responses, and SIF elements which may confer responsiveness to PDGF. The 5' and 3' untranslated regions of the human aggrecan gene were determined to regulate transcription in a promoter and/or cell-type specific manner. The 5'-UTR was found to be stimulatory to the aggrecan promoter, while the 3'-UTR was found to be inhibitory to aggrecan promoter-driven transcription. It was concluded that sequences within the 5'-UTR might bind transcription factors required during the assembly of the transcription initiation complex.

Given the multitude of postranslational modification events during aggrecan biosynthesis, chondrodystophies resulting from mutations in pathways affecting glycosylation, sulfation, and transport, would be expected to exist. One such disorder affecting aggrecan biosynthesis in cartilage has been found to have occurred at the level of sulfate transport. The high incidence of diastrophic dysplasia (DTD) in the Finnish population permitted linkage disequilibrium mapping and identification of a gene responsible for autosomal recessive chondrodysplasia. The gene encodes a sulfate transporter designated DTDST (diastrophic dysplasia sulfate transporter). DTDST mutations have also been detected in patients with a severe recessive form of achondrogenesis, type IB. There is evidence that sulfation of aggrecan is deficient in cartilages in these patients (163,164).

4.2. Link protein gene expression

Rhodes *et al* (165) isolated 5' flanking regions for both the rat and human link protein genes and identified transcriptional start sites. DNA regions necessary for link protein gene expression were examined by transfecting chondrocytes and HeLa cells with plasmid constructs containing the rat link protein promoter coupled to a CAT reporter. Rat and human genomic libraries were screened with oligonucleotides corresponding to 5'cDNA sequence. The 5' flanking sequences for rat and human link protein genes were highly conserved. No consensus TATAA box was observed in either rat or human, but a sequence ACTTAA (a TATAA sequence homolog) was found to occur in the rat sequence. This motif in the human sequence, however, was at the start site of transcription. *Cis* elements included a GC box in the human sequence, and an A-1 and CRE element in the rat sequence. Two regions in the human promoter and one region in the rat had the potential to generate Z-DNA. Repeating purine-pyrimidine bases occurred in two large inverted repeats in both the rat and human sequences, potentially forming large cruciform structures. Comparison of the human and rat promoters revealed two regions having greater than 90% identity. Notably, there was a 12 bp sequence from the link promoter which was also found in the type II collagen promoter, the fibronectin gene and the cartilage matrix protein 5' region. Gel shift analysis of this sequence indicated binding of a sequence-specific protein. Transcriptional activity was analyzed by expression of chimeric CAT plasmids. An 0.85 as well as a 7 kb 5' flanking fragment was found to be transcriptionally active

in chick embryonic chondrocytes, and showed diminished activity in HeLa cells. DNA from the first intron showed enhancer-like activity, in that when it was included with the 7 kb fragment, CAT activity was increased in an orientation-independent manner.

In a more recent study, Rhodes and Yamada (166) further characterized the enhancer-like activity within the first intron of the link protein gene. This activity was found to reside in a 34 bp fragment containing a glucocorticoid-like response element (GRE). Dexamethasone-inducible activity could be reduced by deletion or mutation of this region. Both the rat and homologous human sequence could transfer the ability to respond to dexamethasone and hydrocortisone to a vector containing the thymidine kinase promoter linked to the CAT gene. A second site in the link protein promoter was identified that was required for both glucocorticoid and serum responsiveness. This regions consisted of an AT-rich element, similar to those involved in homeotic protein regulation of the growth hormone gene and the muscle creatine kinase gene. A 32 kDa protein binding to this region was identified in a nuclear extract of chick chondrocytes.

Dudia *et al.* (167) examined genomic organization, determined the transcription start site, and characterized 5' flanking sequence for the human link protein gene. The human link protein gene was found to be greater than 60 kbp in length, and contained five exons corresponding to 5' untranslated region, and each of the structural domains of the protein. In contast to an earlier determination (165), the transcription initiation site was found to be 315 bases upstream from the translation initiation codon. Examination of 5' flanking sequence indicated lack of a TATA box, but a TATAA-like motif (TCTAA) was discovered. The human gene was found to contain a dinucleotide repeat $(CA)_4CT(CA)_{12}$ within the transcribed region.

The chicken link protein gene is extremely large, in excess of 100 kb, and contains five exons that correlate with functional protein domains (168). The LP gene is transcribed from multiple initiation sites between 34 and 76 bp downstream of a TATA-like motif (169). One noteworthy feature of the LP gene is the complex pattern of alternative splicing in the 5' UTR, occurring by differential utilization of six donor and two acceptor splice sites. It was suggested that in the chicken LP gene, where multiple LP mRNA species were seen to arise from the same promoter, gene expression may be further controlled by regulated splicing. Some of the alternatively spliced forms may represent splicing intermediates kept nonfunctional by retaining introns. Since RT-PCR analysis showed cell type and developmental stage specific expression of different LP splice variants, it opens up the intriguing possibility that different splice forms might enable discrete translational control of LP synthesis in different tissues.

4.3. Collagen Type II gene expression

Potential upstream regulatory elements controlling tissue specific transcription of the type II collagen gene (170-176) as well as an enhancer element in the first intron (170) have been described. To delineate *cis*-acting elements of the mouse pro alpha 1(II) collagen gene, Zhou *et al.* (177) demonstrated that a 182 bp intron 1 fragment of the mouse Col2a1 gene was sufficient to direct chondrocye expression in transgenic mice, even when the Col2a1 promoter was replaced with a minimal beta-globin promoter. Further refinement revealed that a minimal 18 bp sequence from the first intron could enhance promoter activity in RCS cells and primary chondrocytes, but not fibroblasts. The 18 bp sequence was found to be part of a larger 48 bp DNA motif that is conserved between human, rat and mouse genes, which contains two inverted repeats of 11 bp each, separated by an 18 bp linker (178).

Sox9 is a member of a family of proteins containing a DNA-binding domain with significant similarity to that of the mammalian SRY (Sex-determining Region Y) gene. A strong correlation between Col2a1 and Sox9 expression in chondrogenic cells was seen (179). Further studies investigated whether Sox9 might contribute to Col2a1 expression. Sox9 was found to bind to the minimal enhancer sequence, and forced expression of Sox9 in nonchondrogenic cells resulted in activation of previously defined chondrocyte-specific Col2a1 enhancer segments. Another study demonstrated that Col2a1was directly regulated by Sox9 *in vivo* (180). In this study, it was shown that mutation of regulatory sequences within the first intron abolished Sox9 binding and chondrocyte-specific expression of a Col2a1-driven reporter gene in transgenic mice. Moreover, ectopic expression of Sox9 could transactivate both a Col2a1-driven reporter gene and the endogenous Col2a1 gene in transgenic mice. Thus, the chondrocyte-specific enhancer in the first intron of the Col2A1 gene was shown to be a direct target for Sox9. These observations are consistent with a previously demonstrated association between mutations in human Sox9 and campomelic dysplasia, a severe skeletal malformation syndrome (181).

In more recent work, a model has emerged in which both Sox9 and other proteins in a DNA-bound complex bind to several HMG-like sites in the first intron to regulate chondrocyte-specific expression. Zhou *et al.* (182) demonstrated that, to confer high level chondrocyte-specific reporter gene expression *in vivo*, the entire 48 bp intronic fragment was required. It was noted that four tandem copies of the 48 bp Col2a1 sequence and 12 tandem copies of an 18 bp element within the 48 bp sequence could act as strong chondrocyte-specific enhancers in transient transfection experiments. However, 12 copies of the 18 bp element showed weaker activity than did 4 copies of the 48 bp enhancer in cartilages of transgenic mice, however. The 48 bp sequence was found to contain multiple *cis*-acting elements essential for *in vivo* chondrocyte-specific expression. Furthermore, chondrocyte-specific nuclear proteins in addition to Sox9 were found to bind to the additional *cis*-acting elements. A number of lines of evidence suggested that other transcription factors in addition to Sox9 were needed to confer high-level COL2A1 expression. For example, Sox9 is highly expressed in Sertoli cells of the

testis (183), a cell which is phenotypically distinct from chondrocytes. This suggests that Sox9 has cell type-specific functions requiring the participation of particular sets of other factors. It was subsequently demonstrated that these additional proteins in chondrocytes, designated CSEPs for chondrocyte-specific enhancer-binding proteins, include a new long form of Sox5 (L-Sox5), and Sox6, two proteins which are members of a Sox sublclass distinct from Sox9 (184).

There may be common mechanisms for regulation of both the Col2a1 and the Col11a2 genes, involving Sox9 and additional proteins bound to related chondrocyte-specific enhancers. Two short chondrocyte-specific enhancer elements have been identified within the mouse Col11a2 promoter (185), a gene predominantly expressed in chondrocytes. Both of these *cis*-elements contain HMG-like sites. They formed a DNA-protein complex containing Sox9 and other proteins, having the same mobility as the Col2a1 complex. The Col11a2 element could activate reporter genes specifically in chondrocytes, and could be activated by Sox9 expression in non-chondrocytic cells. The Col2a1 enhancer in the rat has been delineated in work performed concurrently with the mouse promoter experiments described above (186). In this study the rat Col2a1 enhancer was characterized by determining a minimal sequence length necessary for full enhancer activity. It was demonstrated that a 100 bp segment within the first intron is the minimum size necessary for high level, cell type-specific expression of the Col2a1 gene. Within this region were found several sequence motifs similar to motifs found in the regulatory region of the link protein gene. These motifs included an AT-rich element, a C1 motif and a C3 motif, and two nearly perfect inverted repeat sequences. Constructs containing the 100 bp enhancer region were found to be activated during chondrogenesis when transfected into CFK2 cells. Chondrocyte specific DNA-protein complexes were identified using oligonucleotides from the first intron. Screening by South-Western blotting of a mouse embryonic limb bud cDNA library using oligonucleotide probes from the first intron enhancer region resulted in the identification of the C-propeptide of type II collagen (CPP-II) as an enhancer-binding protein (187). Dnase I footprinting showed that the AT-rich region within the enhancer was protected from digestion, suggesting an interaction between the AT-rich motif and CPP-II.

5. PERSPECTIVE: CHANGES IN CHONDROCYTE GENE EXPRESSION IN OA CARTILAGE

OA pathogenesis is generally considered to be multifactorial. It is a disease with an aging component. OA affects certain individuals more than others and particular joints may show a greater involvement. Furthermore, defined anatomical sites within affected joints may show more pronounced changes. Numerous biomechanical and biological factors, some of which have been discussed in this review, have been found to contribute to disease pathogenesis and progression. The influence of these factors, however, depends upon cellular response, manifested by the normal or altered expression of a subset of genes. There is abundant evidence of altered gene expression in OA cartilage.

In normal articular cartilage, chondrocytes synthesize collagen types II, IX, XI, and VI (188). Chondrocyte precursor (mesenchymal) cells synthesize type I collagen (189). OA chondrocytes have been shown to exhibit increased ECM synthesis in early stages of the disease. There was no alteration in the range of collagen types produced when analyzing solubilized collagen (190), but certain phenotypic changes have been observed in OA cartilage chondrocytes using *in-situ* hybridization techniques. Studies have shown that normal adult articular chondrocytes exhibited very low levels of type II collagen expression consistent with low metabolic activity, but chondrocytes in late stage OA cartilage expressed considerable amounts of alpha 1(II) mRNA (191,192). Furthermore, a subset of chondrocytes in the cartilage superficial and upper middle zone expressing alpha 1(III) but not alpha 1(I) mRNA was observed to overlap with the upper and lower middle zone of chondrocytes showing elevated type II collagen expression. Thus, there may be an "uncoupling" of the usual coordinated pattern of type II, IX and XI collagen expression characteristic of the mature phenotype, in that a subset of chondrocytes was observed to express types II and III simultaneously.

Type X collagen is a normal product of hypertrophic chondrocytes of growth plates (193). Human OA cartilage exhibits pronounced immunostaining for type X collagen surrounding chondrocyte clusters indicating chondrocyte hypertrophy (194). *In-situ* hybridization experiments revealed that clusters which were strongly positive by immunostaining were mostly negative for type X collagen mRNA (195), suggesting a transient expression of type X collagen.

Changes in expression of proteoglycans in OA cartilage have been observed. Proteoglycans in OA cartilage typically is extensively degraded, but shows additional molecular heterogeneity due to new biosynthesis during an intrinsic repair process (196-198). Human osteoarthritic cartilage shows abnormal expression of PG-M (versican) (199), a large chondroitin sulfate proteoglycan which is normally expressed in the prechondrogenic area of chick limb buds (200). Two monoclonal antibodies, 3-B-3 and 7-D-4, have been developed which revealed subtle biochemical changes in proteoglycans in a canine model of OA (201) and in human OA (202). These epitopes appear to be expressed on newly synthesized proteoglycans in OA cartilage, and could reflect OA specific differences in gene expression related to post-translational modification of aggrecan.

Ultimately, it may be that heredity is one of the major factors influencing OA pathogenesis and progression. Genes which may account for the heritable component of OA, however, have not yet been elucidated. Basic science investigations elucidating regulatory

influences on chondrocytes have provided clues to the ultimate identification of relevant "OA genes", the altered expression of which may predispose individuals to OA.

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