BCL-2 REGULATES CHONDROCYTE PHENOTYPE THROUGH MEK-ERK1/2 PATHWAY; RELEVANCE TO OSTEOARTHRITIS AND CARTILAGE BIOLOGY

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By
Rieko Yagi
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Dissertation written by

Rieko Yagi

B.S., Nihon University, 1999

Ph.D., Kent State University, 2005

Approved by

_____________________, Dr. Walter E. Horton Jr.,
Chair, Doctoral Dissertation Committee

_____________________, Dr. John R.D, Stalvey, Doctoral Dissertation Committee

_____________________, Dr. Donna King, Doctoral Dissertation Committee

_____________________, Dr. Gary J. Meszaros, Doctoral Dissertation Committee

_____________________, Dr. Steve Ward, Graduate Faculty Representative

Accepted by

_____________________, Dr. Robert V. Dorman,
Director, School of Biomedical Sciences

_____________________, Dr. John L. West,
Vice President and Dean, Research and Graduate Studies
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CHAPTER ONE

INTRODUCTION

The overview of cartilage and chondrocytes

Cartilage is unique connective tissue that has no direct nervous, blood or lymphatic supply. Significantly, chondrocytes are the only resident cells in cartilage. Thus, the development, maintenance, and repair of cartilage depend largely on chondrocytes. The pathogenesis of osteoarthritis is also associated with phenotype changes in chondrocytes. However, the underlying mechanisms by which chondrocytes maintain a stable phenotype are not completely understood. It is therefore critical to understand how chondrocyte phenotype is regulated in under normal conditions and in age-related degenerative diseases including osteoarthritis.

The undifferentiated mesenchymal cells, i.e. chondrogenic progenitors, initiate aggregation in order to differentiate into matured chondrocytes. This process involves the expression of molecules involved in cell-cell adhesion such as N-cadherin. This cell-cell interaction triggers chondrocyte differentiation. This chondrogenesis process is regulated by multiple growth factors and hormones such as transforming growth factor-β, bone morphogenetic protein, fibroblast growth factor and insulin-like growth factor (Carrington et al., 1991; Geduspan et al., 1993; Kawamura et al., 1988; Murakami et al., 2000a; Yoon et al., 2000; Oh et al., 2003).

Other connective tissue cells are also originated from mesenchyme such as fibroblasts, adipocytes, and osteoblasts. In addition to connective tissue cells, mesenchymal cells develop into smooth muscle cells, and endothelial cells.

There are three fates for the differentiated chondrocytes in cartilage; 1) maintain a stable chondrocyte phenotype such as in articular cartilage, 2) further differentiate into hypertrophic chondrocytes such as during endochondral bone formation, or 3) dedifferentiate into a fibroblast-
like phenotype which may occur in osteoarthritis. The stable phenotype of differentiated chondrocytes is characterized by synthesis of chondrocyte matrix proteins including aggrecan, collagen type II and link protein that are important for tissue function and termed the “cartilage matrix proteins” (Yamada et al., 1991). Collagen type II is exclusively expressed in hyaline cartilage and provides a framework and high tensile strength. Aggrecan, aggregating proteoglycans, is composed of a core protein and many glycosaminoglycan chains that are highly negatively charged and attract water. This nature of aggrecan provides the reversible compressibility and the diffusion of nutrient to cartilage. The tight interaction of aggrecan and hyaluronan, a glycosaminoglycan, is mediated by link protein, which is also proteoglycan. The glycosaminoglycan chains of aggrecan bind to the collagen fibrils, forming a cross-linked matrix.

The differentiated chondrocytes also form the growth plate which is responsible for longitudinal growth of long bones (Yoon et al., 2004). The growth plate is hyaline cartilage where the chondrocytes are organized into five different zones where the cells differentiate and eventually are replaced by bone matrix. The five zones are named resting, proliferative, hypertrophic, calcification and ossification (Fig. 1). Chondrocytes in the resting, proliferative, and prehypertrophic zones of the growth plate express chondrocyte matrix proteins and Sox9, a master chondrocyte transcription factor (Amling et al., 1997; Vornehm et al., 1996; Wang et al., 1997; Yoon et al., 2004; de Crombrugghe et al., 2000). However, in the hypertrophic zone where chondrocytes undergo terminal differentiation and start to express collagen type X and bone related matrix proteins such as osteocalcin and osteopontin, there is decreased expression of cartilage matrix proteins as well as loss of Sox9 expression (Amling et al., 1997; Nerlich et al., 1992; Ng et al., 1997; Zhao et al., 1997).

The phenotype of dedifferentiated chondrocytes is characterized by the loss of chondrocyte matrix protein expression and the expression of collagen types I and III that is
usually expressed by fibroblasts (Sandell et al., 2001). The dedifferentiated chondrocytes are
found in osteoarthritis and in long-term cultured primary chondrocytes (Sandell et al., 2001;
Yoon et al., 2002).

The Sox9 transcription factor binds to regulatory regions of genes coding for chondrocyte
specific matrix proteins such as collagen type II, collagen type XI, aggrecan and cartilage derived
retinoic acid-sensitive protein (Bridgewater et al., 1998; Lefebvre et al., 1997; Sekiya et al., 2000;
Xie et al., 1999). A Sox9 knock out mouse study revealed that Sox9 is also required for
chondrogenesis, chondrocyte differentiation and chondrocyte proliferation (Akiyama et al., 2002;
Bi et al., 1999). Other Sox family transcription factors, Sox5 and Sox6 cooperate with Sox9 to
activate collagen type II expression and chondrocyte differentiation (Lefebvre et al., 2001). Sox9
can be phosphorylated by cAMP-dependent protein kinase A (PKA) which activates its
transcriptional and DNA-binding activity (Huang et al., 2000).

The role of Bcl-2 beyond apoptosis

Bcl-2 is a well-known anti-apoptotic protein normally located in the mitochondrial outer
membrane, endoplasmic reticulum and nuclear envelope (Adams et al., 1998b). Briefly, there are
at least fifteen Bcl-2 family members in mammalian cells, which have either pro- or anti-
apoptotic function. All members posses some of Bcl-2 homology domains (BH1 to BH4). Bcl-2,
an anti-apoptotic protein, prevents the release of cytochrome c that facilitates caspase activity by
creating a heterodimer with Bax, a pro-apoptotic protein, in order to block apoptosis. The active
caspases cleave many death substrates and eventually cause apoptosis.

However, recent accumulating evidence suggests that Bcl-2 regulates other cellular
behavior, in addition to or independent of apoptosis (Biroccio et al., 2000; Chen et al., 1997;
Harada et al., 1998; Haughn et al., 2003; Hilton et al., 1997; Iervolino et al., 2002; Lee et al.,
1998; Matsuzaki et al., 1997; Middleton et al., 1998; Trisciuoglio et al., 2004). For example, the growth and regeneration of retinal or trigeminal axons is promoted by Bcl-2 (Chen et al., 1997; Hilton et al., 1997). Bcl-2 is also necessary for the early maturational change in embryonic sensory neurons (Middleton et al., 1998), and dictates the fate of differentiation of hematopoetic progenitor cells (Haughn et al., 2003; Matsuzaki et al., 1997). Furthermore, the terminal differentiation of keratinocytes is inhibited by Bcl-2 (Harada et al., 1998), and up-regulation of urokinase plasminogen activator receptor expression is induced by Bcl-2 through an extracellular signal-regulated kinase (ERK) signaling pathway that activates Sp1 DNA binding in cancer cells (Trisciuoglio et al., 2004). Bcl-2 overexpression in melanoma cells under hypoxic conditions modulates vascular endothelial growth factor expression (Biroccio et al., 2000; Iervolino et al., 2002). Finally, the activation of the c-Jun N-terminal kinase (JNK) pathway by IL1-β is inhibited by Bcl-2 in an apoptosis-independent manner in fibroblasts (Lee et al., 1998). Although there is a growing consensus that the above functions of Bcl-2 are not entirely a consequence of the anti-apoptotic process, the specific signaling pathways underlying these processes have not been fully identified.

The role of Bcl-2 in chondrocytes

There are reasons to hypothesize that Bcl-2 regulates other cell behavior besides apoptosis in chondrocytes as well as in several other cell types as described above. First, the suppression of Bcl-2 expression leads to accelerated terminal differentiation of chondrocytes and shortened long bones in homozygous knock out mouse (Amling et al., 1997).

Second, the expression of Bcl-2, Sox9 and chondrocyte matrix proteins are co-localized in the growth plate (Fig. 1). Bcl-2, Sox9 and chondrocyte matrix proteins are expressed in resting and proliferating chondrocytes and lost in hypertrophic chondrocytes. Hypertrophic
chondrocytes undergo apoptosis but still have ability to express bone like proteins. Thus, Bcl-2 might be regulating the switch of chondrocyte matrix proteins to bone like proteins in addition to avoid apoptosis. Bcl-2 is also downstream of parathyroid hormone related peptide (PTHrP) (Amling et al., 1997). PTHrP is known to regulate the rate of chondrocyte maturation from prehypertrophic chondrocytes to hypertrophic chondrocytes, and to increase Sox9-dependent collagen type II expression in chondrocytes (Amizuka et al., 1994; Huang et al., 2001; Yoon et al., 2004). From these studies, it is reasonable to suggest that Bcl-2 mediates PTHrP signals in order to have down-stream effects on Sox9. Thus, Bcl-2 might be a regulator of Sox9-dependent matrix expression in growth plate as well as an anti-apoptotic protein that functions to delay hypertrophy.

Third, Bcl-2 expression is decreased in articular cartilage during aging (Kinkel et al., 2003). Aging in cartilage is also associated with increased apoptosis (Adams et al., 1998a), and the decreased matrix production, especially in response to growth factors (Bolton et al., 1999; Buckwalter et al., 1994; Carrington, 2005; Verbruggen et al., 2000). Therefore, the loss of Bcl-2 in aging might be associated with altered chondrocyte phenotype and matrix expression, as well as apoptosis.

Previously we reported that Bcl-2 functions as a regulator of cartilage matrix protein expression in chondrocytes (Feng et al., 1998; Feng et al., 1999; Kinkel et al., 2003). The suppression of Bcl-2 in chondrocytes results in decreased aggrecan expression even when the apoptotic pathway is blocked by inhibition of caspase activity (Feng et al., 1999). Additionally, aggrecan, collagen type II, link protein and Sox9 mRNA expression is significantly decreased in chondrocytes expressing a low level of Bcl-2 (Feng et al., 1999; Kinkel et al., 2003). However, collagen type I mRNA expression is increased suggesting that Bcl-2 may be involved in
regulating the phenotype of chondrocytes (Kinkel et al., 2003). Furthermore, chondrocytes constitutively expressing Bcl-2 are protected not only from apoptosis but also from downregulation of aggrecan, collagen type II and Sox9 following serum withdrawal (Feng et al., 1999; Kinkel et al., 2003). From these studies we hypothesize that Bcl-2 may function to regulate cartilage matrix expression in addition to or independent of its role in regulating apoptosis. However, the precise mechanism by which this occurs is still unknown.
Figure 1. The expression pattern of Bcl-2 and chondrocyte matrix protein in the growth plate. There are five zones of the growth plate; resting, proliferative, hypertrophic, calcification and ossification. Bcl-2 is highly expressed in the proliferative zone where cells also express Sox9 transcription factor, and chondrocyte matrix protein such as collagen type II, aggrecan, and link protein. In contrast, Bcl-2 is decreased or lost in the hypertrophic zone where cells undergo apoptosis. However, the hypertrophic chondrocytes still have the ability to express bone-like protein such as collagen type X, osteopontin and osteocalcin. Thus, Bcl-2 and chondrocyte matrix protein expression is co-localized in the growth plate.
The possible signaling pathways involved in Bcl-2 function in chondrocytes

Three possible signaling pathways; PKCα, NFκB, and MEK-ERK1/2, are proposed to study the link between Bcl-2 and Sox9.

In general, NFκB is activated by cytokines such as TNFα and ILs. The activation of NFκB is caused by release from IκB that is phosphorylated and degraded following the response to cytokines. Murakami et al., (2000b) reported that IL-1 decreases the Sox9-dependent promoter activity through the activation of NFκB in chondrocytes. Moreover, the activation of NFκB suppresses chondrogenesis and destabilizes Sox9 mRNA (Sitcheran et al., 2003). Thus, it is hypothesized that NFκB signaling pathway might negatively regulate the Bcl-2 mediated gene expression (Fig. 2).

PKCα is a member of PKC that is known to be involved in cell proliferation, differentiation, and apoptosis in response to the stimulation of G-coupled protein receptors. PKCα promotes chondrogenesis by activating collagen type II expression, and to block the loss of the differentiated phenotype of chondrocytes (Yang et al., 1998; Yoon et al., 2002). Thus, it is hypothesized that Bcl-2 positively regulates the Sox9-Bcl-2 cascade through PKCα (Fig. 2).

MEK-ERK1/2, in general, is known to regulate differentiation, proliferation, and growth in response to growth factors, mitogens, and stimulation of G-coupled protein receptors. The cascade begins with Ras and Raf kinases (Fig. 2). Ras can be activated by integrins, receptor tyrosine kinases, and calcium etc. The active Ras activates Raf kinase by phosphorylation that phosphorylates ERK1/2 kinase. MEK-ERK1/2 negatively regulates chondrogenesis and chondrocyte matrix protein expression during the differentiation (Bobick et al., 2004; Choi et al., 1995; Murakami et al., 2004; Oh et al., 2000; Seghatoleslami et al., 2003). Thus, it is hypothesized that Bcl-2 positively regulates Sox9 by suppressing the MEK-ERK1/2 cascade.
Figure 2. The possible signaling pathways that regulate Bcl-2 function in regulating matrix gene expression in chondrocytes; PKCα, NFκB, and MEK-ERK1/2. These three possible signaling pathways linking Bcl-2 and Sox9 are tested in chapter two. Bcl-2 might positively regulate Sox9 through the PKCα cascade. The NFκB and MEK-ERK1/2 signaling pathways might be involved as negative regulators of Bcl-2 and Sox9 cascade.
Osteoarthritis

Osteoarthritis (OA) is an age-related degenerative cartilage disease that affects tens of millions of Americans. There is currently no treatment that is effective in curing osteoarthritis, except joint replacement surgery. Many risk factors are considered to contribute to OA, but aging is the major risk factor. The matrix proteins that normally are produced in bone, such as collagen type X, osteocalcin and osteopontin and versican are found in osteoarthritic cartilage (Cs-Szabo et al., 1997; Pullig et al., 2000a; Von der et al., 1992; Eerola et al., 1998; Nishida et al., 1994; Pullig et al., 2000b). These bone proteins alter the biomechanical properties of cartilage. The surface of articular cartilage becomes fibrillated and eventually develops fissures that extend to the deep zone of cartilage. The reported cellular changes such as altered gene expression are not always consistent in different studies because of different tissue resource, age and location in cartilage. The osteoarthritic cartilage is a heterogeneous tissue, so use of entire cartilage can create inconsistent data. Thus, it is critical to understand the basic biology of the disease by elucidating the underlying mechanism using intrajoint comparison of osteoarthritic cartilage.

Hypothesis

The two central hypotheses of the present study are that 1) Bcl-2 regulates Sox9-dependent chondrocyte gene expression and utilizes the specific signaling pathways mediating this function and 2) The expression pattern of Bcl-2 correlates with chondrocyte matrix protein expression in minimal and advanced osteoarthritis cartilage.

1. **Bcl-2 regulates Sox9-dependent chondrocyte gene expression and utilizes the specific signaling pathways mediating this function.**

   The following five specific hypothesis were tested to address the first central hypothesis.
a. The level of Bcl-2 modulates Sox9-dependent chondrocyte matrix gene expression. This hypothesis is addressed by analyzing changes of Sox9-dependent promoter activity or chondrocyte matrix protein transcription in several different condition. The Bcl-2 level was manipulated in few ways such as Bcl-2 siRNA transient transfection, sense Bcl-2 adenovirus infection in primary rat chondrocytes, or stable sense or antisense Bcl-2 transfection in Immortalized Rat Chondrocytes (IRC cells). Sox9-dependent collagen type II promoter activity was decreased in presence or absence of a caspase inhibitor when the Bcl-2 level was suppressed. The Sox9-dependent promoter activity and chondrocyte matrix transcription were maintained in cells constitutively expressing Bcl-2 following serum withdrawal that causes apoptosis and decrease Bcl-2 expression in chondrocytes as well as many other cell types. Therefore, it is concluded that Sox9-dependent chondrocyte matrix gene expression depends on the level of Bcl-2.

b. The NFκB pathway is involved in the maintenance of chondrocyte phenotype by Bcl-2. NFκB is known to suppress Sox9-dependent promoter activity and destabilize Sox9 mRNA during chondroogenesis (Sitcheran et al., 2003). NFκB activity was examined in wild type and sense Bcl-2 IRC cell lines with serum withdrawal. A higher NFκB activity was found in sense Bcl-2 cell lines than in wild type IRC cell lines. Serum withdrawal does not influence Sox9-dependent promoter activity in any cell lines tested. However, co-transfection with Sox9-dependent reporter construct and an IκB dominant negative vector revealed that the inhibition of NFκB dose not affect Sox9 activity. Thus, it is concluded that NFκB is not involved in the Bcl-2 function to modulate the Sox9-dependent promoter activity.
c. Bcl-2 regulates chondrocyte phenotype through a PKCα signaling pathway.

PKCα is known to positively regulate collagen type II expression during chondrogenesis (Yang et al., 1998; Yoon et al., 2002). The pattern of phosphorylated PKCα was determined and indicated that the phosphorylation was decreased only in wild type IRC cells with serum withdrawal. The basal level of phosphorylation was higher in sense Bcl-2 cell lines compared with wild type IRC. However, it was concluded that PKCα phosphorylation does not mediate the Bcl-2 regulated matrix expression based on the use of two different PKCα inhibitors.

d. The MEK-ERK1/2 cascade negatively regulates chondrocyte phenotype. A MEK inhibitor increased Sox9-dependent promoter activity and transcription of matrix proteins as well as proteoglycan accumulation in the pericellular matrix in wild type chondrocytes. Thus, the MEK-ERK1/2 pathway limits the expression of chondrocyte matrix proteins in matured chondrocytes.

e. Bcl-2 regulates chondrocyte morphology and matrix gene expression through MEK-ERK1/2 cascade. The increased activation of ERK1/2 was observed in two different antisense Bcl-2 cell lines compared with wild type cells. The antisense Bcl-2 cell lines are characterized by the fibroblastic morphology, the loss of chondrocyte matrix proteins and expression of collagen type I, which is normally expressed by fibroblasts. The inhibition of MEK-ERK1/2 cascade reversed the fibroblastic phenotype to chondrocytic phenotype as well as increased chondrocyte matrix expression and accumulation of proteoglycan in pericellular matrix.
2. **The expression pattern of Bcl-2 correlates with chondrocyte matrix protein expression in minimal and advanced human osteoarthritis cartilage.**

   The following three specific hypothesis were tested to address the second central hypothesis.

   **a. The morphology of advanced OA cartilage is distinctively different from minimal OA cartilage.** The advanced OA cartilage was obtained from areas with in 1cm of overt lesions and the minimal OA cartilage was obtained from areas with no obvious surface defects. The histological and biochemical data suggest that advanced OA cartilage is distinct from minimal OA cartilage.

   **b. The mRNA expression pattern of Bcl-2 and chondrocyte matrix protein in advanced OA cartilage is distinct from in minimal OA cartilage.** The mRNA expression of osteopontin is significantly increased in advanced OA cartilage compared with minimal OA cartilage. In contrast, chondrocyte matrix protein mRNA expression is decreased in advanced OA cartilage compared with minimal OA cartilage.

   **c. The mRNA expression pattern of Bcl-2 correlates with Sox9 and aggregan in advanced OA relative to in minimal OA.** A correlation between Bcl-2, Sox9 and aggregan mRNA expression was found using the entire data set. Thus it is concluded that Bcl-2 might regulate chondrocyte matrix protein expression in osteoarthritis as well as in mature chondrocytes.
CHAPTER TWO

THE REGULATION OF CARTILAGE MATRIX PROTEIN GENE EXPRESSION BY BCL-2

INTRODUCTION

The anti-apoptotic protein Bcl-2 has critical functions, in addition to or independent of
apoptosis in variety of cell types as described detail in chapter one (Biroccio et al., 2000; Chen et
al., 1997; Harada et al., 1998; Haughn et al., 2003; Hilton et al., 1997; Iervolino et al., 2002; Lee
et al., 1998; Matsuzaki et al., 1997; Middleton et al., 1998; Trisciuoglio et al., 2004). However, a
major question how Bcl-2 influences other cellular behavior besides anti-apoptotic function is
still not answered. Previous works in our laboratory have provided evidence that Bcl-2 functions
as a regulator of Sox9-dependent cartilage matrix protein expression in chondrocytes as described
in chapter one (Feng et al., 1998; Feng et al., 1999; Kinkel et al., 2003). However, further studies
are needed to see the direct effect of Bcl-2 in chondrocytes, since immortalized rat chondrocyte
cell lines stably transfected with sense or antisense Bcl-2 are used for these previous experiments.
The stable transfection may cause secondary effects. Thus, Bcl-2 siRNA, sense Bcl-2
adenovirus, and primary rat articular chondrocytes are used to elucidate the direct Bcl-2 function
in matrix protein regulation. In addition to the Sox9 transcription, Sox9-dependent collagen type
II promoter activity by Bcl-2 was also determined. We hypothesize that Bcl-2 may function to
regulate Sox9-dependent chondrocyte matrix expression in addition to or independent of its role
in regulating apoptosis through the specific signaling pathways.

Here, the three possible pathways are tested to be involved in the regulation of matrix gene
expression by Bcl-2: PKCα, NFκB and ERK1/2. PKCα positively regulates expression of
collagen type II during chondrocyte differentiation, and is down-regulated during
dedifferentiation (Chang et al., 1998; Yang et al., 1998; Yoon et al., 2000). NFκB inhibits Sox9 activity, destabilizes Sox9 mRNA, and decreases collagen type II expression with TNFα treatment (Murakami et al., 2000b; Sitcheran et al., 2003). The ERK1/2 signaling pathway is known to negatively regulate collagen type II, aggrecan and Sox9 mRNA in mesenchymal cells prior to chondrocyte differentiation (Bobick et al., 2004; Seghatoleslami et al., 2003). Conversely, the ERK1/2 pathway has been shown to positively regulate Sox9 mRNA and activity during chondrogenesis induced by fibroblast growth factor (Murakami et al., 2000a). In general, the pattern of expression of active PKCα is directly correlated with differentiation and collagen type II expression, while ERK1/2 expression is inversely correlated, even though PKCα and ERK appear to regulate chondrogenesis independently (Yoon et al., 2002). The findings presented here support a novel role for Bcl-2 in regulating the differentiated phenotype of chondrocytes. In addition, the data support a model where by Bcl-2 suppresses an inhibitory action of the MEK-ERK1/2 signaling pathway on Sox9-dependent regulation of chondrocyte gene expression downstream of Bcl-2.
METHODS

Cell Culture

Primary chondrocytes were isolated from femoral condyles of 6-day-old Sprague-Dawley rats (Charles River). Cartilage was digested with 0.4% (w/v) collagenase (Worthington Biochemical Corporation) in a shaking incubator at 37 °C for one hour. The cells were recovered by centrifugation at 4 °C. The cell pellet was washed with PBS two times and cells were seeded on dishes in Ham’s F-12 medium (Gibco BRL) with 10% FBS. Immortalized rat chondrocytes (IRC) were cultured in Ham’s F-12 medium with 10% FBS. The IRC cells are known to have a differentiated phenotype similar to articular chondrocytes (Horton, Jr. et al., 1988). Cells were transfected with plasmids containing sense or antisense Bcl-2 coding sequences and selected in G418 as described previously (Feng et al., 1998; Feng et al., 1999). The cells were plated at a density of $1 \times 10^6$ in T25 flasks for protein isolation or $3.85 \times 10^5$ in 6-well pates for RNA extraction and were cultured at 37°C with 5% CO$_2$ in Ham’s F-12 medium.

Adenoviral Vectors and Infection in Chondrocytes

The adenovirus vectors were generated with the adeno X-expression system (BD Clontech). The recombinant adenovirus vector expressing rat Bcl-2 cDNA was constructed using the identical sequence coding for Bcl-2 as previously described (Feng et al., 1998). Adenoviral vectors containing the luciferase cDNA (Adeno-x-LacZ, BD Clontech) was used as control vector. The purified recombinant adenoviral DNA containing the coding region in the sense orientation for Bcl-2 or LacZ was transfected into HEK293 cells and recombinant adenovirus was produced. The viral stocks were concentrated and the purified stock was titered using an Adeno-X Rapid Titer Kit (BD Clontech). The infection efficiency of adenovirus, as determined by â-
galactosidase staining (Invitrogen), was 100% with 30 MOI in IRC cells and 45 MOI in primary chondrocytes. Primary chondrocytes or IRC cells were plated at a density of $6 \times 10^5$ in 6-well plates. 45 MOI or 30MOI recombinant virus diluted with 500ul OptiMEM (Gibco) was added to cells and incubated for 90 min. Next, 1ml of complete medium was added to each well and following incubation for an additional 6 h, the virus-containing medium was replaced by complete culture medium.

**Antibodies and Other Reagents**

Antibodies recognizing total ERK1/2 and phosphorlylated ERK1/2 were purchased from Cell Signaling. Antibodies to phosphorylated PKCα and β-actin were obtained from Santa Cruz Biotechnologies. Anti-Bcl-2 was obtained from Transduction Laboratories. For immunofluorecence staining, the primary Bcl-2 antibody and anti-rabbit FITC secondary antibody were purchased from Santa Cruz Biotechnologies. The general caspase inhibitor, ZVAD-fmk, was purchased from Alexis and used at concentration of 50µM that has been determined to block apoptosis in IRC chondrocyte cell lines (Feng et al., 1999). The MEK1/2 inhibitor, U0126 (Cell Signaling), was used at concentrations of 20, 25, 35µM depending on experiments. The PKCα inhibitors, Go6983 (Martiny-Baron et al., 1993) and GF109203X (Toullec et al., 1991) (Sigma) were used at concentrations of 1µM and 5µM which have been shown to suppress PKCα activity (Kim et al., 2002; Yoon et al., 2002).

**Western Blotting**

Cells were lysed and scraped in RIPA buffer consisting of 20mM Tris (pH 7.9), 140mM NaCl, 5mM EDTA, 1mM EGTA, 10mM NaF, 1% Nonidet P-40, 1% Triton X-100, 10% glycerol, 1µg/ml aprotinin, 10mM phenylmethylsulfony fluoride, 1mM Na$_2$VO$_4$, and phosphatase
inhibitor cocktail I (Sigma). The cell lysate was incubated on ice, and centrifuged at 14,000 × g for 15 min at 4°C. The protein concentration was determined by BCA protein assay (Pierce). 15-40µg of total protein was mixed with Tris-Glycine SDS sample buffer and sample reducing agent (Invitrogen) and loaded on a 7.5% or 10% SDS-PAGE gel. Following electrophoresis, the proteins were transferred to PVDF membranes (Invitrogen). The membranes were blocked in Tris-buffered saline with 0.1% Tween and 5% non-fat milk for 1 h at room temperature and incubated overnight at 4°C with primary antibody. After washing with TBS-T, membranes were incubated with secondary antibody for 1 h at room temperature. The membranes were developed with chemiluminescence reagents (Pierce). The expression level of positive bands of proteins were quantified using Image Station 440CF (Kodak) and normalized to the level of expression of β-actin.

**Transient Transfection, β-galacosidase and Luciferase Assay**

Cells were plated at 3 × 10^5 in 12-well plates 24 h prior to transfection. A construct containing four repeats of a Sox9 binding site with the collagen type II promoter driving the luciferase reporter gene (Lefebvre et al., 1996; Lefebvre et al., 1997), (generous gift from Dr. V. Lefebvre, Cleveland Clinic Foundation) or an NFκB reporter construct (Stratagene) was co-transfected with a β-galactosidase reporter vector as an internal control of transfection efficiency. The dominant negative IκB expression vector (Stratagene), or Bcl-2 or control siRNA (IMGENEX) were also co-taransfected with the Sox9 construct and β-galactosidase reporter vector. All transfections were performed with Lipofectamine2000 (Invitrogen) mixed with OpitMEM (Gibco). After the appropriate amount of incubation time, cells were lysed with Reporter Lysis Buffer (Promega). β-galactosidase activities were determined by measuring
absorbance at 420nm and Luciferase activities were determined by the Luciferase Assay System (Promega) using Lumat LB 9501/16 luminometer (Berthold).

**Immunofluorescence staining**

Cells were seeded at $1.4 \times 10^5$/well in 4 well chamber slides and transfected with Bcl-2 or control siRNA expression vectors (IMGNEX). 48h after transfection, cells were fixed with 5% formalin for 30min, incubated with 0.2% Triton X, and then blocked with 2% Goat Serum. Next, cells were incubated overnight with the Bcl-2 primary antibody at 4°C and then incubated with FITC secondary antibody. DAPI mounting medium (Vector) was applied, the slides were coverslipped and fluorescence was observed with immunofluorescence microscope BX60 (Olympus).

**The analysis of sulfated cartilage matrix proteoglycan**

After treatments as indicated, cells were fixed and stained with 0.5% Alcian Blue 8GX, pH 1.0 as described previously (Hassell et al., 1982). The Alcian Blue bound to matrix proteoglycan was extracted with 4M guanidine hydrochloride and measured at 600nm (Chang et al., 1998).

**Quantitative real-time PCR**

Total RNA was isolated using Trizol reagent (Invitrogen) and treated with DNase (Invitrogen) to remove genomic DNA. Next, 1µg of total RNA was reverse transcribed to cDNA using TaqMan reverse transcription reagents (Applied Biosystems) following the manufacturer’s protocol. The aggrecan, collagen type II, Sox9 and 18S primers (Table 1) were designed and examined for primer efficiency as described previously (Kinkel et al., 2003). Briefly, primers
were designed using Primer Express software (Applied Biosystems) and primer efficiency was performed with a standard curve of 100, 50, and 5ng cDNA with each primer compared to 18S. The specificity of amplified products was confirmed by dissociation curve analysis (Applied Biosystems). The quantitation of mRNA expression was performed using the Applied Biosystems ABI Prism 7700 sequence detection system (Applied Biosystems). The PCR reactions were performed using 50ng cDNA and SYBR Green PCR core reagents (Applied Biosystems) in 96-well plates following the manufacturer’s protocol. The data were analyzed using Sequence Detector v1.7 software (Applied Biosystems). Relative expression was calculated using the Comparative C<sub>T</sub> Method (User Bulletin #2, Applied Biosystems, and reference, (Kinkel et al., 2003). According to accepted standards, relative differences of 2-fold or greater are considered as biologically significant (Applied Biosystems).

**Statistical Analysis**

The statistical analysis for transient transfection was performed using Mann-Whitney test. To compare multiple samples, row data was analyzed by one-way analysis of variances with Dunnett multiple comparison post-tests.

The statistical analysis for relative mRNA expression by quantitative real time PCR was performed using Mann-Whitney test.
<table>
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RESULTS

The level of Bcl-2 protein modulates Sox9-dependent chondrocyte matrix gene expression.

The previous data from our laboratory demonstrated that the suppression of Bcl-2 protein level with integrated antisense constructs resulted in down regulation of expression of mRNA coding for cartilage matrix proteins even in the presence of caspase inhibitors that blocked full apoptosis (Feng et al., 1998; Feng et al., 1999; Kinkel et al., 2003). Here we examine the effects of direct down regulation of Bcl-2 using siRNA on Sox9-dependent promoter activity. IRC cells were transfected with a plasmid vector expressing siRNA for Bcl-2 along with the Sox9 construct containing luciferase. Immunocytochemistry was used to demonstrate no change in Bcl-2 level in IRC chondrocytes transfected with control siRNA containing random DNA sequences (Fig. 3A), while there was a significant decrease in the percentage of chondrocytes expressing Bcl-2 following transfection with the Bcl-2 siRNA (Fig. 3B).

The Sox9-dependent reporter construct contains four copies of the Sox9 binding site upstream from a minimal collagen II promoter (Lefebvre et al., 1996; Lefebvre et al., 1997). This reporter construct is dependent on transcriptional activation by Sox9 in IRC chondrocytes since mutations in the Sox9 binding sites eliminate promoter activity (data not shown). The Sox9 dependent reporter activity was decreased by 70% in chondrocytes with suppressed Bcl-2 expression (Fig. 4A). The down-regulation of Sox9-dependent promoter activity by loss of Bcl-2 was not recovered in the presence of 50µM ZVAD, a general caspase inhibitor to block apoptosis (Fig. 4B). These results confirm that Sox9-dependent promoter activity is dependent on Bcl-2 in chondrocytes.

A specific role for Bcl-2 was further established by expressing Bcl-2 using an adenoviral construct prior to exposure of the chondrocytes to Bcl-2 siRNA. Cells were first infected with
either sense Bcl-2 or LacZ adenovirus, and then co-transfected with either Bcl-2 or control siRNA along with the Sox9 activity reporter construct. Cells infected with LacZ virus and transfected with Bcl-2 siRNA showed a 50% downregulation of Sox9-dependent promoter activity compared with control siRNA (Fig. 5). However, if the cells were first treated with Bcl-2 adenovirus in order to increase base-line level of Bcl-2, there was no decrease in Sox9 dependent promoter activity following transfection with Bcl-2 siRNA (Fig. 5).
Figure 3. The suppression of Bcl-2 by Bcl-2 siRNA.
IRC cells were transiently transfected with 0.5µg of Bcl-2 siRNA or control siRNA plasmid. After 48h incubation, immunofluorescence was performed to detect Bcl-2 protein using FITC. Nuclei were stained with DAPI (blue). (A) IRC chondrocytes transfected with control siRNA plasmid showed strong expression of Bcl-2 in all cells. (B) A representative field of IRC chondrocytes transfected with Bcl-2 siRNA showing loss of Bcl-2 expression.
Figure 4. The down-regulation of Sox9-dependent collagen type II promoter activity by Bcl-2 siRNA. The IRC chondrocytes were transiently co-transfected with a Sox9-dependent reporter construct and Bcl-2 or control siRNA along with β-galactosidase as an internal control for transfection efficiency. (A) Sox9-dependent promoter activity was suppressed by Bcl-2 siRNA to 30% of control siRNA at 48h after transfection. (B) After transfection, cells were treated with 50µM ZVAD, a potent caspase inhibitor for 48h. The suppression of promoter activity was not recovered with 50uM ZVAD in Bcl-2 siRNA transfected cells compared with control siRNA transfected cells. The relative promoter activities are mean of ± S.D. of three or five independent experiments. Statistical significance was determined by Mann-Whitney test; *** P< 0.001, compared to control.
Figure 5. Sox9-dependent promoter activity depends on the level of Bcl-2. IRC chondrocytes were first infected with LacZ or Bcl-2 adenovirus (30MOI) and then transfected with Bcl-2 or control siRNA, the Sox9-dependent reporter construct and the β-galactosidase expression plasmid. The Sox9-dependent promoter activity level was not down-regulated by Bcl-2 siRNA in cells containing high basal level of Bcl-2 protein (adeno-Bcl-2), whereas LacZ adenovirus infected cells showed a 50% decrease in Sox9 activity following transfection with Bcl-2 siRNA. The relative promoter activities are mean of ± S.D. of three or five independent experiments.
Previously we demonstrated that constitutive expression of Bcl-2 protected IRC chondrocytes against serum withdrawal-induced down regulation of mRNA transcripts coding for several cartilage matrix proteins and Sox9 (Kinkel et al., 2003). Here we extend these studies to look directly at Sox9-dependent promoter activity. The Sox9 reporter construct was co-transfected with a β-galactosidase expression vector into wild-type IRC chondrocytes and two independent IRC lines (S-1 and S-2) that constitutively express Bcl-2 (Feng et al., 1998). After transfection the cells were exposed to serum withdrawal for 24 and 48 hours. The Sox9-dependent promoter activity was decreased by 50% and 75% at 24 and 48 hours respectively in wild-type IRC chondrocytes (Fig. 6). However, there was no loss of Sox9 dependent promoter activity following serum withdrawal in the two cell lines with constitutive Bcl-2 expression (Fig. 6).

Finally, we established a protective role for Bcl-2 in maintaining the differentiated phenotype of primary chondrocytes. Primary rat chondrocytes were infected with adenoviral vectors expressing LacZ or Bcl-2. At 24 hours after infection, cells were exposed to serum withdrawal for 24 hours and the steady state level of collagen type II, aggrecan, and Bcl-2 mRNA was determined (Fig. 7). Adeno-LacZ infected cells showed decreased steady state levels of mRNA transcripts coding for collagen type II, aggrecan, Sox9 and Bcl-2 with serum withdrawal compared to cells cultured in medium with 10% FBS. The protein level of Bcl-2 was found to be decreased with serum withdrawal in cells infected with adeno-LacZ but not with adeno-Bcl-2 (data not shown). In contrast, primary rat chondrocytes infected with adeno-Bcl-2 were protected from the effect of serum withdrawal and, in fact, generally had elevated levels of all four mRNA transcripts (Fig. 7).
Figure 6. The constitutive expression of Bcl-2 protects against down-regulation of Sox9-dependent promoter activity following serum withdrawal. The Sox9 dependent reporter construct and ã-galactosidase plasmid were transfected into wild type IRC chondrocytes or two independent IRC lines constitutively expressing Bcl-2 (S-1 and S-2). All cells were serum deprived for 24 and 48hr. The activity of Sox9 was suppressed with serum withdrawal in wild type IRC chondrocytes, whereas cells expressing a high level of Bcl-2 maintained the Sox9 activity. The relative promoter activities are the mean ± S.D. of three to five independent experiments. Statistical significance was determined by Mann-Whitney test among three different cell lines; * P< 0.05.
Figure 7. Expression of mRNAs coding for cartilage matrix protein and Bcl-2 in primary chondrocytes infected with LacZ or sense Bcl-2 adenovirus followed by serum withdrawal. After infection with adenovirus (45MOI), the rat primary chondrocytes were serum-deprived for 24hr. Quantitative real time PCR was performed to detect the expression of collagen type II, aggrecan, Sox9 and Bcl-2 mRNA. The relative expression of all four mRNA transcripts was decreased following serum withdrawal in cells infected with the LacZ control adenovirus. In contrast, serum withdrawal did not result in down-regulation of mRNA transcripts in cells exposed to adeno-Bcl-2. The relative mRNA expression is the mean ± S.D. of three independent experiments.
The NFκB pathway is not involved in the maintenance of chondrocyte phenotype by Bcl-2.

The down-regulation of Sox9-dependent promoter activity by IL-1 involves activation of NFκB (Murakami et al., 2000b). In addition, NFκB has been shown to be involved in the suppression of chondrogenesis and destabilization of Sox9 mRNA (Sitcheran et al., 2003). Therefore, we considered the possibility that NFκB might be down stream of Bcl-2 in regulating Sox9-dependent chondrocyte gene expression. A vector containing four repeats of an NFκB binding site, TATA box, and a luciferase reporter was transfected into wild type IRC cells and two independent IRC cell lines with stable expression of Bcl-2. The cells were cultured for 24 hours in the presence or absence of serum. Serum withdrawal did not change the NFκB activity in wild type IRC cells (Fig. 8A) even though Sox9-dependent promoter activity was significantly decreased under the same condition (Fig. 6). The Sense Bcl-2 cell lines also did not show any effect of serum withdrawal on NFκB activity. However, the level of NFκB activity in IRC cell lines with constitutive expression of Bcl-2 was three-fold higher than in wild type cells (Fig. 8A). This suggested a possible role for high basal activity of NFκB in the protective effect of Bcl-2 on Sox9-dependent promoter activity. Therefore, we used a dominant negative IκB expression vector, co-transfected with the Sox9 reporter construct and a β-galactosidase expression construct to test this hypothesis. The dominant negative IκB expression vector effectively blocked NFκB activity in both wild type and sense Bcl-2 IRC cell lines (data not shown). However, the forced inactivation of NFκB by dominant negative IκB did not change Sox9-dependent promoter activity level compared with the empty vector control DNA in wild type or sense Bcl-2 IRC cell lines cultured in 10% FBS (Fig. 8B). These data do not support a role for NFκB in the pathway by which Bcl-2 regulates chondrocyte gene expression.
Figure 6. The NFκB pathway is not directly involved in the Bcl-2 regulation of Sox9-dependent collagen type II promoter activity. (A) Wild type or sense Bcl-2 IRC cell lines were transiently transfected with NFκB reporter construct and β-galactosidase plasmid and then exposed to serum withdrawal for 48hr. NFκB activity was not changed with serum withdrawal compared to 10% FBS in wild type and sense Bcl-2 IRC lines, whereas two sense Bcl-2 cell lines showed a 3-fold higher level of NFκB activity with or without serum, compared to wild type cells. (B) Wild type IRC chondrocytes were transiently transfected with a dominant negative IκB expression vector and Sox9-dependent reporter construct. No effect of dominant negative IκB on Sox9 dependent promoter activity was observed. The relative promoter activities are mean ± S.D. of three independent experiments. Statistical significance was determined by one-way analysis of variances with Dunnett multiple comparison post-test; *** P< 0.001.
**Bcl-2 regulation of chondrocyte phenotype is not dependent on PKCα.**

PKCα has been shown to promote chondrogenesis by activating collagen type II expression, and to block the loss of the differentiated phenotype of chondrocytes (Yang et al., 1998; Yoon et al., 2002). The level of phosphorylated PKCα is decreased in wild type IRC cells cultured in 0% FBS compared to cells cultured in 10% FBS (Fig. 9). In contrast, sense Bcl-2 cell lines showed a high level of phosphorylated PKCα and this level was maintained following serum withdrawal (Fig. 9). Further, two independent antisense Bcl-2 IRC cell lines showed decreased PKCα phosphorylation compared with wild type cells (data not shown). The data suggests that PKCα might be important for mediating the effect of Bcl-2 on regulating the differentiated phenotype of chondrocytes.

In order to test this hypothesis, wild type and IRC lines with constitutive expression of Bcl-2 were treated with two different PKCα inhibitors, Go6983 and GF109203X, and transfected with the Sox9-dependent promoter reporter construct. Both inhibitors resulted in decreased Sox9 dependent promoter activity in wild type IRC cells compared to cells treated with vehicle (Fig. 10A). However, neither inhibitor decreased Sox9-dependent promoter activity in the IRC lines with constitutive expression of Bcl-2 cultured in medium with or without serum (Fig. 10B). These results suggest that PKCα is not required for the Bcl-2 dependent pathway that maintains Sox9 activity.
Figure 9. The phosphorylation of PKCα in wild type and sense Bcl-2 cell lines (S-1 and S-2) with serum withdrawal. Chondrocytes were incubated for 24 hr in the presence or absence of 10% serum. The phosphorylation of PKCα was determined by densitometry following Western blot analysis and then normalized to the actin protein level as a loading control. Lane 1, wild type IRC in 10% FBS; lane 2, wild type IRC in 0% FBS; lane 3, S-1 IRC in 10% FBS; lane 4, S-1 IRC in 0% FBS; lane 5, S-2 IRC in 10% FBS; lane 6, S-2 IRC in 0% FBS. Serum withdrawal suppressed the phosphorylation of PKCα in wild type IRC, whereas phospho-PKCα remained high in sense Bcl-2 cell lines. The relative phospho-PKCα protein levels are represented as the mean ± S.D. of four independent experiments. Statistical significance was determined by Mann-Whitney test; ** P < 0.01.
Figure 10. The effect of PKCα inhibitors, Go6983 and GF109203X, on Sox9-dependent collagen type II promoter activity. (A) After transfection of the Sox9-dependent reporter construct, cells were treated with 1 µM or 5 µM Go6983 (Go) or GF109203X (GF), or 0.1% or 0.5% Me2SO vehicle alone for 24h. The Sox9 activity was depressed following treatment with two different inhibitors in wild type IRC cells. (B) Neither PKCα inhibitor depressed Sox9-dependent promoter activity in the Bcl-2 sense lines in the presence or absence of serum. The relative promoter activities are shown as the mean ± S.D. of three independent experiments. Statistical significance was determined by one-way analysis variance with Dunnett multiple comparison; ** P< 0.01; * P< 0.05.
MEK-ERK cascade negatively regulates chondrocyte phenotype.

Both Sox9-dependent promoter activity and collagen type II expression have been shown to be negatively regulated through the MEK-ERK pathway during chondrogenesis (Bobick et al., 2004; Choi et al., 1995; Murakami et al., 2004; Oh et al., 2000; Seghatoleslami et al., 2003). We examined this pathway in wild type IRC chondrocytes following serum withdrawal. Wild type IRC cells expressed abundant total ERK1/2 and a low level of phosphorylated ERK1/2 (Fig. 11A). Serum withdrawal for 24 hours resulted in strongly increased phosphorylation of ERK1/2, but the total ERK1/2 level was unaffected (Fig. 11A). We next used the MEK inhibitor, U0126, to determine if the increased ERK phosphorylation with serum withdrawal was related to decreased chondrocyte gene expression. However, it proved difficult to block the high level of phospho-ERK1/2 (and presumably the high level of MEK activity) following serum withdrawal (data not shown). Therefore, we blocked the basal level of phosphorylated ERK1/2 in wild type IRC cells cultured in 10% FBS in order to determine if ERK1/2 was acting as a negative regulator of chondrocyte phenotype in the IRC model. The MEK inhibitor, U0126 resulted in a clear decrease in phospho-ERK1/2 levels in IRC cells cultured in 10% FBS (Fig. 11B). Note that the level of exposure of the blot is relatively long in Fig. 9B in order to clearly document the downregulation of phosphorylated ERK1/2 following treatment with the inhibitor.

The inhibition of phosphorylated ERK1/2 resulted in a strong upregulation of the steady state level of mRNA coding for aggrecan, collagen II, and Sox9 (Fig. 12A) as well as an increase in the Sox9-dependent promoter activity (Fig. 12B). These data suggest that ERK1/2 functions in a pathway that negatively regulates expression of genes coding for cartilage matrix proteins in differentiated IRC chondrocytes. This finding was confirmed using Alcian Blue, a metachromatic dye that stains the proteoglycan component of the cartilage matrix (Hassell et al., 1982). Treatment of the wild type IRC cells with U0126 resulted in increased accumulation of
Alcian Blue positive matrix compared to vehicle alone even in IRC cells cultured in medium containing 10% FBS (Fig. 13).
Figure 11. The phosphorylation of ERK1/2 in wild type IRC cells with serum withdrawal. (A) After 24h serum withdrawal, the level of phosphorylated and total ERK1/2 protein was determined. The phosphorylation of ERK1/2 was dramatically increased following 24h of serum withdrawal. (B) The level of phosphorylated and total ERK1/2 protein was determined in IRC chondrocytes cultured in media containing 10% serum treated with 0.2% Me₂SO or 20µM U0126. The endogeneous phosphorylation of ERK1/2 was suppressed with U0126, whereas total ERK1/2 protein level remained unaffected.
Figure 12. The effect of endogeneous phosphorylated ERK1/2 in wild type IRC cells on chondrocyte matrix protein mRNA expression and Sox9-dependent promoter activity. (A) Wild type IRC chondrocytes were treated with 0.2% Me₂SO or 20µM U0126 treatment for 24h, and mRNA was extracted and quantitative real time PCR was performed. The mRNA coding for aggrecan (Agg), collagen type II (Col2), and Sox9 were up-regulated 2-3 fold following inhibition of phospho-ERK1/2. (B) After transient transfection of Sox9-dependent reporter construct, IRC chondrocytes were treated with 0.2% Me₂SO or 20µM U0126 for 24 and 48hr. U0126 induced a greater than 2 fold upregulation of Sox9 activity. The data shown in panels A and B represent typical results obtained from three to four independent experiments. The relative mRNA expression and the relative promoter activity are the mean ± S.D. of three independent experiments.
Figure 13. The effect of inhibition of phospho-ERK1/2 on the accumulation of cartilage matrix proteoglycan in wild type IRC chondrocytes. IRC chondrocytes were stained with Alcian Blue that binds sulfated proteoglycan in the matrix, following 24h incubation in medium containing 0.2% Me₂SO or 20µM U0126. (A) 0.2% Me₂SO as control. (B) 20µM U0126. Treatment with U0128 increased Alcian Blue staining indicating increased proteoglycan in pericellular matrix. Shown are representative data from three independent experiments.
Bcl-2 regulates chondrocyte morphology and matrix gene expression through a pathway involving the MEK-ERK cascade.

In order to test the hypothesis that the ERK1/2 pathway is downstream of Bcl-2, we utilized the antisense Bcl-2 IRC lines and Bcl-2 siRNA. We previously demonstrated that the steady state level of mRNA coding for aggrecan, collagen II, and Sox9 were all down regulated in IRC lines following knock down of Bcl-2 with antisense constructs (Kinkel et al., 2003). The level of phospho-ERK1/2 but not total ERK1/2 is upregulated in two different antisense Bcl-2 cell lines consistent with a role in the negative regulation of the differentiated chondrocyte phenotype (Fig. 14A). Inhibition of MEK1/2 using the U0126 inhibitor resulted in a concentration-dependent suppression of phospho-ERK1/2 in the AS-8 line (Fig. 14B, Lane 1-4). A non-specific effect of Me₂SO resulted in partial suppression of phospho-ERK1/2 levels at the highest concentration of the vehicle (Fig. 14B, Lane 3). However, treatment with the MEK inhibitor at this concentration resulted in a complete loss of phopho-ERK1/2 expression (Fig. 14B, Lane 4). The inhibition of the MEK-ERK pathway resulted in a reversal of the fibroblastic phenotype observed for the antisense Bcl-2 line and an accumulation of cartilage matrix proteoglycan as measured by Alcian Blue staining (Fig. 15). The untreated IRC antisense Bcl-2 lines displayed a fibroblastic morphology with little accumulation of Alcian Blue positive matrix (Fig. 15A). However, the same cell line treated with 35μM of U0126, which strongly inhibited the production of phospho-ERK1/2, displayed a chondrocyte phenotype with abundant Alcian Blue positive matrix (Fig. 15B). The same increase in Alcian Blue positive matrix was observed in another independent antisense Bcl-2 cell line treated with 35μM U0126. In addition, a lower dose of 25μM U0126 also resulted in increased matrix accumulation in both antisense Bcl-2 cell lines (data not shown). Consistent with the increase in the accumulation of extracellular matrix, the steady state level of mRNA transcripts for collagen II, aggrecan and Sox9 were upregulated with the MEK inhibitor,
U0126. Specifically, both concentrations of the MEK inhibitor increased the expression of mRNA coding for collagen type II and Sox9 three to five-fold (Fig. 16A). However, only the higher concentration of U0126 produced an upregulation of aggrecan mRNA (Fig. 16A). Finally, the relationship between phospho-ERK1/2 levels and Sox9-dependent promoter activity was examined following knock down of Bcl-2 expression with siRNA. Treatment of cells receiving Bcl-2 siRNA with the Me₂SO control did not prevent inhibition of Sox9-dependent promoter activity. However, inhibition of the MEK/ERK pathway with U0126 maintained promoter activity even in the absence of Bcl-2 (Fig. 16B). There was some non-specific protection of Sox9-dependent promoter activity with 0.35% of Me₂SO vehicle alone compared with the 0.25% concentration, but this non-specific effect was still less than that observed with the 35µM concentration of U0126. Specifically, 35µM U0126 induced a three-fold upregulation of Sox9 activity in cells transfected with Bcl-2 siRNA compared with control siRNA. These results support a model in which Bcl-2 suppresses the inhibitory action of the MEK-ERK1/2 signaling pathway on Sox9-dependent regulation of chondrocyte gene expression.
**Figure 14.** The phosphorylation of ERK1/2 in antisense Bcl-2 IRC cell lines. (A) The two independent antisense Bcl-2 cell lines (AS-3 and AS-8) showed high level of phosphorylated ERK1/2 compared with wild type IRC cells, whereas total ERK1/2 level was stayed same. (B) Antisense Bcl-2 cell line was treated with 25µM (Lane 2) and 35µM (Lane 4) U0126. Both concentrations of the MEK inhibitor decreased the level of phosphorylated ERK1/2 in the antisense Bcl-2 IRC line. The low level of Me₂SO vehicle alone (0.25%, Lane 1) did not affect the level of Phosphorylated ERK1/2, whereas the higher concentration (0.35%, Lane 3) showed some non-specific inhibition. The data shown represent typical results obtained from three independent experiments in two different antisense IRC cell lines that showed equivalent results.
Figure 15. The effect of U0126 on the accumulation of cartilage matrix proteoglycan in antisense Bcl-2 IRC cell lines. Cells were stained with Alcian Blue that binds sulfated proteoglycan in matrix, following 48h incubation in medium containing 0.35% Me₂SO (A) or 35μM U0126(B). U0126 reversed the fibroblastic phenotype to a chondrocytic phenotype and dramatically increased Alcian Blue staining in the pericellular matrix. The data shown represent typical results obtained from three independent experiments.
Figure 16. The effect of the MEK inhibitor on cartilage matrix protein mRNA expression and Sox9 activity in antisense Bcl-2 or cells transfected with Bcl-2 siRNA. (A) Cells were treated with 25µM or 35µM U0126, or 0.25% or 0.35% Me₂SO as a control for 24h. The mRNA level of aggregcan (Agg), collagen type II (Col2), and Sox9 was increased with the MEK inhibitor in antisense Bcl-2 cell line. A higher dose of U0126 was required to stimulate aggregcan mRNA expression compared to collagen type II and Sox9. (B) The wild type IRC cells were co-transfected with the Sox9-dependent reporter construct and Bcl-2 siRNA expression vector. After transfection, cells were incubated with medium containing 25µM or 35µM U0126, or 0.25% or 0.35% Me₂SO. Sox9-dependent promoter activity was upregulated in siRNA transfected chondrocytes following treatment with the MEK inhibitor. Note that the higher dose of vehicle did attenuate the down regulation of Sox9 activity following treatment with Bcl-2 siRNA. Representative data from two different cell lines showed equivalent data. The mean ± S.D. was calculated from three to four independent experiments.
DISCUSSION

The present study is an extension of our previous work demonstrating that Bcl-2 has a regulatory role in maintaining the phenotype of differentiated chondrocytes, independent of the traditional and well established anti-apoptotic effect of Bcl-2 (Feng et al., 1998; Feng et al., 1999; Kinkel et al., 2003). Here we demonstrate, for the first time, that Bcl-2 modulates Sox9-dependent chondrocyte gene expression through suppression of the MEK-ERK1/2 pathway.

There is now an increasing body of data supporting our previous study that suggests a role for Bcl-2, other than that of regulating the mitochondrial pathway of apoptosis as described in chapter one (Biroccio et al., 2000; Chen et al., 1997; Harada et al., 1998; Haughn et al., 2003; Hilton et al., 1997; Iervolino et al., 2002; Lee et al., 1998; Matsuzaki et al., 1997; Middleton et al., 1998; Trisciuoglio et al., 2004). Briefly, Haughn et al. recently reported that Bcl-2 and Bcl-X\textsubscript{L} have novel roles in directing hematopoetic cell differentiation, in addition to controlling cell survival (Haughn et al., 2003). Others also report that Bcl-2 inhibits terminal differentiation of keratinocytes by decreasing expression of keratin10/11, a differentiation marker (Harada et al., 1998). The overexpression of Bcl-2 in cancer cells exposed to hypoxia induces urokinase plaminogen activator receptor (uPAR) expression through increased Sp1 transcriptional activity induced by the ERK1/2 pathway, while antisense Bcl-2 expressing cells have decreased uPAR protein expression (Trisciuoglio et al., 2004). However, with the exception of the last study listed above, there is no information published on possible signaling pathways underlying the effects of Bcl-2 on gene expression patterns that define the differentiated phenotype of cells.

Our first goal was to provide a foundation for identifying possible signaling pathways related to Bcl-2 regulation of differentiated chondrocyte phenotype by undertaking a series of experiments using primary and immortalized chondrocytes. First, we demonstrated that knock-
down of Bcl-2 expression with siRNA resulted in decreased activity of the Sox9 transcription factor, in addition to a decreased level of Sox9 mRNA as previously reported (Kinkel et al., 2003b). This effect was independent of apoptosis because suppression of Sox9 activity by Bcl-2 siRNA was not prevented by treatment with an inhibitor of caspase activity that blocks full apoptosis. Since siRNA can have non-specific effects (Persengiev et al., 2004; Scacheri et al., 2004), and to show the specificity of our observation, we used an alternative technique and demonstrated that increasing the baseline level of Bcl-2 using an adenoviral based vector attenuated the loss of Sox9 activity caused by transfection of Bcl-2 siRNA. Herein, we also report that independent clonal IRC chondrocyte lines constitutively expressing Bcl-2 are protected from loss of Sox9 activity following serum withdrawal. Because immortalized cell lines may have different properties than the primary cell from which they were derived, we utilized primary rat chondrocytes to demonstrate the protective effect of maintaining a high Bcl-2 level on the expression of genes coding for Sox9, as well as major cartilage matrix proteins. Together, these findings supported further studies to characterize the signaling pathways operating in chondrocytes that may mediate the Bcl-2-dependent regulation of the differentiated phenotype.

Our second goal was to identify specific signaling pathways mediating the regulation of Sox9-dependent chondrocyte gene expression by Bcl-2. There are several potential signaling pathways likely to be downstream of Bcl-2 in the regulation of Sox9-dependent gene expression in chondrocytes, including NFκB, PKCα, and MEK-ERK1/2. NFκB has been shown to either promote or block apoptosis depending on the stimulus (Li et al., 2002; Varfolomeev et al., 2004). In case of chondrocytes, NFκB is known to inhibit Sox9 activity, destabilize Sox9 mRNA, and decrease collagen type II expression with TNFα treatment (Murakami et al., 2000b; Sitcheran et al., 2003). In the present study, we observed that NFκB activity was not changed with serum
withdrawal in wild type IRC cells, even though Sox9-dependent promoter activity was decreased. However, the basal level of NFκB activity was higher in IRC chondrocyte lines expressing increased and constitutive levels of Bcl-2 compared to wild type IRC chondrocytes. These observations led us to hypothesize that NFκB may be involved in maintaining Sox9 activity and chondrocyte specific gene expression. However, this hypothesis could not be supported because, although a dominant negative IκB expression vector transfected into chondrocytes reduced NFκB activity, there was no effect on Sox9-dependent promoter activity in either wild type or IRC-Bcl-2 lines. This suggests that NFκB is not required for the Bcl-2 mediated Sox9 dependent promoter activity. In fact, studies have implicated NFκB signaling pathway as a negative regulator of Sox9 activity and collagen type II mRNA expression following TNFα treatment (Murakami et al., 2000b; Sitcheran et al., 2003). It is possible that the elevated Bcl-2 in the IRC lines is able to overcome any potential negative regulation by NFκB.

Because it has been reported that PKCα is a positive regulator of chondrogenesis (Chang et al., 1998; Choi et al., 1995; Yoon et al., 2002), and that the PKCα signaling pathway is also involved in chondrocyte matrix protein expression and in the maintenance of the differentiated chondrocyte phenotype (Yoon et al., 2002), we hypothesized that PKCα was possibly downstream of Bcl-2 as a positive regulator of chondrocyte matrix gene expression. This hypothesis was supported by the finding that the PKCα level declined with serum withdrawal in wild type IRC chondrocytes but not in IRC cell lines with constitutive expression of Bcl-2. In addition, we demonstrated a positive role for PKCα in maintaining the differentiated phenotype in wild type IRC cells using inhibitors of PKCα activity that suppressed Sox9 activity. Our data suggest that PKCα is a positive regulator of Sox9-dependent collagen type II expression (Fig. 10A) and proteoglycan synthesis (data not shown). Previous work using a mesenchymal cell
model, showed that the level of PKCα increased during chondrogenesis (Yoon et al., 2002). However, in that study the inhibition of PKCα did not result in loss of collagen type II expression, suggesting that other factors were important for maintaining the phenotype in differentiated mesenchymal cells. In the present study, exposure of IRC lines with constitutive expression of Bcl-2 to PKCα inhibitors (in the presence or absence of serum) did not result in loss of Sox9 transcriptional activity, even at concentrations higher than those that were active in wild type cells. This significant finding suggests that Bcl-2 expression, above a critical level, overcomes the dependence of PKCα for maintaining Sox9-dependent gene expression in chondrocytes. In addition, this observation points to another signaling pathway that might be downstream of Bcl-2. Specifically and importantly, a recent report demonstrated that IGF-1 induces chondrogenesis and blocks dedifferentiation through two different mechanisms namely, by activating PKCα and inhibiting ERK 1/2 (Oh et al., 2003).

The ERK1/2 signaling pathway is known to act as a negative regulator of chondrogenesis of mesenchymal cells to chondrocytes, and also suppresses the expression of collagen type II, aggrecan, and Sox9 (Bobick et al., 2004; Chang et al., 1998; Oh et al., 2000). Other studies have suggested that in response to specific growth factors such as FGF-2, BMP and TGF-β, the MEK-ERK pathway is a positive signal for chondrocyte gene expression (Murakami et al., 2000a; Seghatoleslami et al., 2003; Watanabe et al., 2001). However, it is clear that a negative regulatory role is operating in the IRC cells, as demonstrated by the low basal level of phosphorylated ERK 1/2 in wild type cells cultured in 10% serum, as compared to the increased level observed following serum withdrawal. Interestingly, the present study shows that inhibition of the low level of ERK1/2 in the wild type cells actually upregulates the steady state level of mRNA coding for aggrecan, collagen II, and Sox9, as well as Sox9 activity, similar to what has been reported with increased expression of Bcl-2 (Kinkel et al., 2003b). It was also striking that
the already differentiated IRC cells could be induced to accumulate even more Alcian blue positive extracellular matrix. However, it is likely that much of the matrix secretion was lost into the culture medium (Bobick et al., 2004), so our results probably underestimate the increase in matrix synthesis. It is important to note that this finding is similar to that reported earlier using PD98059, another MEK inhibitor, which increased collagen type II and proteoglycan protein synthesis (Yoon et al., 2002). However, U0126 used in the current study is a more specific MEK inhibitor (Davies et al., 2000). Collectively, our data suggests that endogenous phosphorylation of ERK1/2 serves as a limiting regulator of Sox9 dependent cartilage matrix protein expression.

The observed relationship between the level of phospho-ERK1/2 and the differentiated phenotype of IRC cells led us to look for a link between Bcl-2 and the MEK-ERK 1/2 pathway. First, in two independent clonal IRC lines with suppressed Bcl-2 expression, secondary to integration of an antisense Bcl-2 construct, ERK1/2 was highly phosphorylated. It has been shown previously that these antisense Bcl-2 lines have lost the differentiated chondrocyte phenotype (Feng et al., 1998; Feng et al., 1999; Kinkel et al., 2003). In the present study, we used a MEK inhibitor to up-regulate the steady state level of mRNA coding for aggrecan, collagen II, and Sox9, as well as to stimulate Sox9 activity in the antisense Bcl-2 lines. In addition, we demonstrated that the fibroblastic phenotype of the antisense Bcl-2 IRC chondrocytes was reversed with inhibition of MEK and that the cells accumulated abundant Alcian Blue positive extracellular matrix. Collectively, these findings indicate that the suppressive action of the MEK-ERK signaling inhibited by Bcl-2 pathway on Sox9-dependent gene expression in chondrocytes. These findings are consistent with the recent reports by Trisciuoglio et al showing that Bcl-2 regulates ERK signaling in cancer cells under hypoxic conditions (Trisciuoglio et al., 2004). In addition, Pardo et al demonstrated that the ERK signaling pathway regulates Bcl-2 expression by fibroblast growth factor-2 as part of an apoptosis
pathway, suggesting some feedback regulation (Pardo et al., 2002). Future work will be directed at further defining the specific cellular pathway linking Bcl-2 and MEK-ERK1/2 in maintaining the phenotype of chondrocytes.

There is a physiological and pathological role for Bcl-2 in regulation of chondrocyte phenotype, not only in *in vitro*, as reported here, but importantly in *in vivo* as well. The PTHrP receptor, Bcl-2 and Sox9 are highly expressed in prehypertrophic chondrocytes in the growth plate but the expression is lost in hypertrophic chondrocytes (Amling et al., 1997; Huang et al., 2000; Iwamoto et al., 1994; Lee et al., 1996). Overexpression of PTHrP in mice or upregulation of PTHrP in chondrocytes results in increased Bcl-2 protein and inhibition of differentiation from prehypertrophic to hypertrophic chondrocytes (Amling et al., 1997). Together, these studies and our previous work suggest that Bcl-2 acts to maintain the chondrocyte phenotype in growth plate chondrocytes by increasing collagen type II and Sox9 activity, as well as inhibiting apoptosis. This action of Bcl-2 is likely to be an important part of the mechanism by which the Indian Hedgehog/PTHrP feedback loop delays the chondrocyte differentiation from proliferative chondrocytes to hypertrophic chondrocytes (Amling et al., 1997). This report is consistent with the finding that PTHrP maintains the chondrocyte phenotype by increasing Sox9 activity and phosphorylation of Sox9 by cAMP-dependent protein kinase A (Huang et al., 2000; Huang et al., 2001). Also, it has been reported that PTHrP and Bcl-2 knockout mice as well as a Sox9 conditional knockout mouse model all have shortening of long bones (Akiyama et al., 2002; Amling et al., 1997). We recently reported that there is a correlation between the expression of Bcl-2, Sox9 and aggrecan in chondrocytes located in human osteoarthritic articular cartilage as will be described in chapter three (Yagi et al., 2005b). Furthermore, inhibition of the MEK-ERK pathway can prevent the progression of osteoarthritis by maintaining the chondrocyte phenotype (Pelletier et al., 2003). Thus, clearly, there is *in vivo* significance with respect to the signaling
pathway by which Bcl-2 regulates the chondrocyte phenotype during development, normal aging and disease.

Our finding that Bcl-2 regulates Sox9-dependent chondrocyte gene expression through suppression of the MEK-ERK1/2 cascade strongly suggests a physiologically important link between Bcl-2 and MEK-ERK1/2 signaling. This role of Bcl-2, as a regulator of gene expression through MEK-ERK1/2, has also been found in cancer cells (Trisciuoglio et al., 2004). Thus, the Bcl-2 regulation of important cell processes besides anti-apoptotic function through the MEK-ERK1/2 might be a general model in many cell types.
CHAPTER THREE
INTRAJOINT COMPARISONS OF GENE EXPRESSION PATTERNS
IN HUMAN OSTEOARTHRITIS

INTRODUCTION

Osteoarthritis (OA) is a progressive, debilitating disease that involves changes in the cartilage, bone and surrounding soft tissue of diarthrodial joints. Although the major risk factor for developing OA is aging, the exact mechanisms underlying OA pathogenesis are poorly understood (Karlson et al., 2003; Miranda et al., 2002). The characterization of pathogenic mechanisms that are important for OA progression would allow for the development of therapies designed to delay the need for arthroplasty and extend the useful life of the natural joint. However, because of ethical limitations, undertaking such a task in a longitudinal manner in individual human patients is not feasible. Currently, it is common to compare advanced OA cartilage from joint replacement procedures to non-OA cartilage obtained at autopsy or from trauma cases. These samples may come from individuals that are not matched according to age, genetic background or other factors. Several studies by Aigner’s group have utilized normal cartilage and early degenerative cartilage obtained from donors within 24-48 hours of death compared with late stage OA cartilage from patients undergoing joint replacement surgery (Bau et al., 2002a; Gebhard et al., 2003; Aigner et al., 2001; Soder et al., 2002; Bau et al., 2002b). The major findings reported from these studies include; a higher level of expression of both matrix metalloproteinase (MMP) 13 and ADAM-TS5 in the OA cartilage (Bau et al., 2002a), up-regulation of mRNA for several collagen types in advanced OA compared to early stage OA and normal cartilage (Aigner et al., 2001; Gebhard et al., 2003), alterations in type VI collagen distribution and appearance in OA cartilage (Soder et al., 2002), and no change in the expression...
level of Smads in OA versus normal cartilage (Bau et al., 2002b). Another study compared gene expression profiles between normal cartilage obtained from patients undergoing joint replacement following femoral neck fracture or obtained after foot amputation with OA cartilage obtained following knee, hip or ankle joint arthroplasty (Martin et al., 2001). In contrast to previous studies, the higher ratio of collagen II/collagen I and aggrecan/versican mRNA expression was found in normal cartilage compared to OA cartilage. Finally, an increased level of osteopontin at both the protein and mRNA level has been reported in cartilage obtained from patients undergoing knee arthroplasty compared to non-OA cartilage obtained at autopsy (Pullig et al., 2000a). These studies are important, however, it is important to consider additional sampling protocols that help address confounding variables such as age, genetics and postmortem changes.

Recently, a sampling method that compares lesional and non-lesional cartilage from adjacent areas of the same joint was reported (Shlopov et al., 1997; Shlopov et al., 2000; Squires et al., 2003; Cole et al., 2003; Mollenhauer et al., 2002; Price et al., 2002). This approach has great potential but further studies are needed to fully exploit this methodology. The present study extends this approach by carefully defining the gross and histological features of the cartilage and determining gene expression patterns that include the relationship between mRNA transcripts coding for Bcl-2 and for cartilage matrix proteins.

Chondrocytes express the highest level of Bcl-2, anti-apoptotic protein, along with major matrix proteins in the prehypertrophic zone of the growth plate (Amling et al., 1997; Vornehm et al., 1996b; Wang et al., 1997a). As chondrocytes mature and undergo hypertrophy, Bcl-2 expression is lost (Amling et al., 1997). Bcl-2 is also required to maintain the chondrocyte matrix proteins and Sox9 transcription factor in cells isolated from articular cartilage (Feng et al., 1999; Kinkel et al., 2003). However, the relationship between Bcl-2, Sox9 and matrix gene expression has not been examined using an in vivo human cartilage system. Thus, the objective of this study
was to first test the hypothesis that patient matched minimal and advanced OA cartilage would show significant differences in cell and matrix characteristics. The second objective was to begin to examine differential patterns of gene expression in this model and to specifically test the hypothesis that decreased Bcl-2 expression would be associated with altered Sox9 and matrix gene expression *in vivo*. 
METHODS

**Cartilage samples**

Human cartilage samples were removed from femoral condyles of patients, ranging in age from 65-83 years, within 3 hours of knee joint replacement surgery, and immediately stored at -80 °C for subsequent RNA extraction. Only general information about the patients such as age and gender were provided. The collection procedure was reviewed by the NEOUCOM IRB and considered exempt from full review. Representative samples were fixed in 10% buffered formalin for 24hr for histological analysis. Each sample was initially categorized, based on its gross morphology, as either 1) advanced OA cartilage, which was taken from areas within 1 cm of overt lesions located on the medial condyle, or 2) minimal OA cartilage taken from areas with no obvious surface defects on the lateral condyle (Fig. 17). Full thickness cartilage specimens were obtained.

**Scoring disease severity**

After fixation the cartilage samples were embedded in paraffin and 6 µm-thick sections were stained with 0.1% Thionin (Fisher Biologics) which is a metachromatic stain that principally identifies the glycosaminoglycan component of cartilage. A precise scoring system was used to objectively quantify the disease severity based on four categories: fibrillation, chondrocyte cloning, matrix depletion and cellularity (Table 2). Each scoring category was analyzed separately and compared between minimal and advanced OA cartilage. The stained sections were analyzed using Bioquant Nova v5.00.8 software (R&M Biometrics, Nashville, TN).
Figure 17. Representative location of minimal and advanced OA cartilage. (A) Distal femur of patient undergoing knee arthroplasty showing full thickness degeneration of cartilage in medial femoral condyle (MFC) and relatively normal appearance of cartilage in lateral femoral condyle (LFC). (B) Appearance of cartilage obtained from the area surrounding the lesion in the medial femoral condyle. (C) Appearance of cartilage obtained from the lateral femoral condyle.
### Table 2. Scoring system for evaluation of progressing OA

<table>
<thead>
<tr>
<th>Table 2. Scoring system for evaluation of progressing OA</th>
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<tbody>
<tr>
<td><strong>Fibrillation</strong></td>
</tr>
<tr>
<td>1. Even surface</td>
</tr>
<tr>
<td>2. Uneven surface</td>
</tr>
<tr>
<td>3. Fibrillation on only superficial zone</td>
</tr>
<tr>
<td>4. Fissures in mid-zone</td>
</tr>
<tr>
<td>5. Fissures in deep zone</td>
</tr>
<tr>
<td><strong>Chondrocyte cloning</strong></td>
</tr>
<tr>
<td>1. No clusters</td>
</tr>
<tr>
<td>2. Chondrocyte clusters in superficial zone (No greater than 4 cells)</td>
</tr>
<tr>
<td>3. Chondrocyte clusters beyond superficial zone (No greater than 4 cells)</td>
</tr>
<tr>
<td>4. Chondrocyte clusters of more than 4 cells</td>
</tr>
<tr>
<td><strong>Matrix Depletion</strong></td>
</tr>
<tr>
<td>% of thickness of non-metachromatic area to entire area</td>
</tr>
<tr>
<td><strong>Cellularity</strong></td>
</tr>
<tr>
<td>The number of cells in non-metachromatic staining area and in metachromatic staining area</td>
</tr>
</tbody>
</table>
Biochemical analysis

The proteoglycan content of the cartilage was determined as previously described (Chen et al., 2003). Briefly, the cartilage samples were desiccated and digested with 120µg/ml proteinase K. The sulfated glycosaminoglycan concentration was determined using dimethylmethylene blue (de Jong et al., 1994) and hydroxyproline concentration was determined using a standard protocol (Reddy et al., 1996).

Isolation of RNA from human OA cartilage and preparation of cDNA

All articular cartilage samples were evaluated histologically to confirm the degree of cartilage degeneration and then the articular cartilage was ground using a freezer mill (Spex PertiPrep, Metuchen, NJ) and homogenized with Trizol reagent (Gibco BRL). The supernatant of the homogenate was mixed with 70% ethanol and applied to RNeasy Midi column (Qiagen) and the RNA was isolated following the suggested protocol. The quality of the RNA was determined by electrophoresis through an agarose gel and the RNA was treated with DNase (Gibco BRL). 0.5µg of total RNA was reverse transcribed to cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol.

Primer design and verification

MMP3, MMP8, MMP9 and MMP13 probes were obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems). Primers for 18S, Collagen type II, Sox9 and Bcl-2 (Table 3) were designed using Primer Express software (Applied Biosystems) and synthesized by Fisher Scientific (Pittsburgh, PA). The specificity of the primers was confirmed by BLAST. 18S was used as an internal control to normalize the amount of RNA in each sample, as described in User Bulletin #2 (Applied Biosystems). The primer efficiency was performed with a standard
curve of 60, 6, and 0.6 ng cDNA with each gene compared to 18S. The specificity of amplified
gene products was confirmed by melting curve analysis.

**Quantitative real time PCR**

The quantitation of gene expression was performed using the Applied Biosystems ABI
Prism 7700 sequence detection system (Applied biosystems). PCR reactions were performed in
triplicates using 30 ng cDNA and SYBR Green PCR core reagents (Applied Biosystems) for
primers or Taq Man Universal PCR master mix (Applied biosystems) for probes in 96-well plates
following the manufacturer’s protocol. The data were analyzed using Sequence Detector v1.7
software (Applied Biosystems). Relative expression was calculated using the Comparative C\text{T}
Method (User Bulletin #2, Applied biosystems, and reference (Kinkel et al., 2003).

**Statistical Analysis**

The statistical significance was determined by the Mann-Whitney test using relative
mRNA expression, with values of p<0.05 considered significant. The correlation data was
analyzed using Spearman’s correlation coefficient.
Table 3. Sequence of primers for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence</th>
<th>Concentration (nM)</th>
<th>Gene Bank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>Forward</td>
<td>5'-AGTCCCTGCCCTTTGTACACA-3'</td>
<td>300</td>
<td>X03205</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GATCCGAGGGCCTCCTAAAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggrecan</td>
<td>Forward</td>
<td>5'-TGCATCCACGAAGCTAACCT-3'</td>
<td>50</td>
<td>XM007701</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGCCTCGCCTTTGAAAT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen Iía</td>
<td>Forward</td>
<td>5'-TGGTCTTGGTGAAACTTTGC-3'</td>
<td>50</td>
<td>NM001844.3</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GCCCATTTTGCCTTTGACTTA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Forward</td>
<td>5'-CGCCCTGTGGATGACTGAGTA-3'</td>
<td>50</td>
<td>NM000657</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CTACCCAGCTCCGTTATCCT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sox9</td>
<td>Forward</td>
<td>5'-CAGTACCGCGACTCGCAAA-3'</td>
<td>300</td>
<td>NM000346</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CTCGTGGAGAAGTCTCCAGACCT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Link Protein</td>
<td>Forward</td>
<td>5'-TGCTGGAAAAATTTCTCGGATA-3'</td>
<td>100</td>
<td>NM001884</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GGCGCTTCCCTGGACTAGA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Forward</td>
<td>5'-TGCTAAAAACCTGACCCATCT-3'</td>
<td>50</td>
<td>NM000582</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GCTTTCGTGGACTTACTGGAA-3'</td>
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</table>
RESULTS

Characterization of minimal and advanced OA cartilage

The initial hypothesis of the present study was that human cartilage specimens obtained from the same patient could be divided into minimal and advanced samples based on objective criteria. An example of a typical histological appearance of tissue exhibiting minimal and advanced disease is shown in Fig. 18. Minimal disease tissue (Fig. 18A) displayed a uniform distribution of chondrocytes throughout the width and depth of the tissue and had a high content of chondroitin sulfate proteoglycan, as evidenced by the extensive metachromatic staining of the cartilage matrix. There was variable disruption of the joint surface and less metachromasia in the superficial zone compared to the deeper zones. Advanced OA tissue showed histological characteristics that clearly distinguished it from minimal OA tissue (Fig. 18B, C and D). In general, in advanced OA cartilage, the superficial zone was absent and deep clefts (fissures) extended through the mid-zone into the deep zone. The chondrocytes were distributed in a non-uniform pattern of focal clusters interspersed among non-cellular regions of cartilage. The degree of metachromasia was less pronounced compared to minimal cartilage within both the territorial and interterritorial matrix.

The histological differences between minimal and advanced OA cartilage were objectively quantified using a standardized scoring system (Table 2) and the Bioquant image analysis software and was found to be statistically significant for the fibrillation score (Fig. 19A). In addition, the extent of chondrocyte cloning was scored, as chondrocytes may actively proliferate during attempted repair and form clusters with four or more cells. Advanced OA cartilage showed a statistically significant increase in the cloning score compared to minimal OA cartilage (Fig. 19B). The amount of proteoglycan was assessed by staining with Thionin and...
measuring the percent area of non-metachromasia compared to total area. There was a statistically significant loss of metachromatic staining in the advanced OA cartilage compared to the minimal OA cartilage (Fig. 19C). Finally, there was no observed difference in total cellularity observed between minimal and advanced OA cartilage in either the metachromatic or non-metachromatic areas (Fig. 19D).
Figure 18. Typical histology observed with minimal and advanced OA cartilage. (A) Minimal OA cartilage showing an even surface, uniform distribution of chondrocytes, and intense metachromasia. (B-D) Examples of advanced OA cartilage are shown. Important features include chondrocyte cloning (arrows), surface fissures (asterisks), area of nonmetacromatic statining (nm) indicating loss of proteoglycan, and metachromatic staining (m). The sections were stained with Thionin, which is a metachromatic stain that principally identifies the glycosaminoglycan content of cartilage.
Figure 19. Quantitative assessment of disease progression in minimal and advanced OA cartilage. (A) Fibrillation (n=15). (B) Cloning (n=15). (C) Percentage of proteoglycan depletion (n=13). (D) Cell density (n=13). The statistical significant was found in fibrillation, cloning and percentage of proteoglycan depletion. The overall cell density was higher in non-metachromatic staining area than in metachromatic staining area. However, there was not difference between minimal and advanced OA cartilage. Statistical analysis was performed using paired t-test. ***p<0.001, **p<0.01.
**Biochemical study**

The content of glycosaminoglycan (GAG) and hydroxyproline was examined in specimens of advanced and minimal OA cartilage (Fig. 20). The GAG content was found to be statistically significantly lower in advanced OA compared to minimal OA cartilage (Fig. 20A). This finding is consistent with the loss of metachromasia observed in the advanced OA cartilage. The content of hydroxyproline did not change significantly in advanced and minimal OA cartilage (Fig. 20B). The hydroxyproline content is a general indicator of the overall collagenous content of the cartilage but will not distinguish between specific collagen sub-types or intact versus cleaved collagen.

**Relative gene expression from matched cartilage samples**

Based on the histological and Bioquant analysis data, there was a clear difference in tissue and cellular characteristics in minimal versus advanced OA cartilage obtained at arthroplasty. Next, we examined whether there were alterations in expression patterns of nine critical genes, aggrecan, collagen type II, Bcl-2, Sox9, Link protein, osteopontin, and MMP-13, -3, and –9 (Fig. 21). A direct patient-matched comparison using all patients studied revealed a statistically significant increase in the steady-state level of osteopontin mRNA in advanced OA cartilage compared to minimal cartilage (3.58 ±1.42, p < 0.01, Fig. 21A). In addition, clear trends were observed in the expression patterns of aggrecan and link protein (Fig. 21A). Specifically, the mRNA expression levels of aggrecan and link protein were significantly decreased in advanced OA cartilage relative to minimal OA cartilage (respectively 0.63±0.6, p<0.05 and 0.629±0.45, p<0.05, Fig. 21A). The steady-state level of mRNA coding for collagen type II displayed a wide-range of variation in the minimal versus advanced OA cartilage. Of the ten patients studied, three exhibited increased collagen type II expression, three had the same  


expression level in minimal and advanced OA cartilage, and four showed decreased expression in advanced OA cartilage relative to minimal OA cartilage.

We next examined the expression pattern of mRNA coding for two regulatory molecules, Bcl-2 and Sox9 (Fig. 21B). Seven of the patients had decreased Bcl-2 mRNA expression in advanced OA cartilage compared to minimal OA cartilage. The remaining four patients showed similar or slight increased levels of Bcl-2 mRNA in advanced OA compared to minimal OA cartilage. The expression of Sox9 mRNA showed a similar pattern. The expression of mRNA coding for MMPs was variable with regard to the disease states (Fig. 21C). Five out of eight patients showed a decreased MMP13 mRNA expression in advanced compared to minimal OA cartilage, while two patients showed no change and one patient showed increased expression. Similarly, MMP9 and MMP3 mRNA expression was decreased in advanced OA cartilage in the majority of patients. However, a small subset of patients showed an elevated expression of mRNA coding for MMP3 and MMP9 in advanced OA cartilage compared to minimal OA cartilage.
Figure 20. Glycosaminoglycan (GAG) and hydroxyproline content. Quantitative analysis of the levels of GAG, n=5 (A) and hydroxyproline, n=5 (B). dry weight (dw). The decline in glycosaminoglycan content in advanced OA cartilage compared minimal OA cartilage indicates down-regulation aggrecan protein. The hydroxyproline content showed no change in advanced OA compared with minimal OA cartilage. Statistical analysis was performed using Mann-Whitney. *p<0.05 as compared to minimal OA cartilage.
Figure 21. Relative expression of steady state mRNA coding for matrix proteins, regulatory proteins, and MMPs in advanced OA cartilage. The expression level of each mRNA transcript is shown for advanced OA cartilage relative to minimal OA cartilage, which is set at 1 (dotted line). (A) Aggrecan (Agg, n=11), collagen type II (Col2, n=10), link protein (Link, n=10) and osteopontin (Osteo, n=8) expression level. (B) Bcl-2 (n=11) and Sox9 (n=11) expression level. (C) MMP13 (n=8), MMP3 (n=9), and MMP9 (n=9) expression level. Most gene expression was decreased in advanced OA cartilage compared with minimal OA cartilage. Osteopontin was strongly up-regulated in most of patients except one. Collagen type II did not show any trend.
Correlation between Bcl-2, Sox9, aggrecan and collagen type II expression

The second hypothesis tested was that the Bcl-2 mRNA level would correlate with both the Sox9 and aggrecan mRNA levels in vivo as previously reported in vitro (Kinkel et al., 2003). We used the entire data set to look for correlated patterns of mRNA expression for aggrecan, Bcl-2, Sox9, and collagen type II mRNA by comparing expression levels in advanced OA cartilage compared to minimal OA cartilage. Figure 22A shows a statistically significant positive correlation between Bcl-2 and aggrecan mRNA expression in advanced OA compared with minimal OA (Spearman r = 0.83, p=0.002). Bcl-2 and Sox9 mRNA expression also showed a statistically significant positive correlation (Fig. 22B, Spearman r = 0.69, p=0.013). However, while a trend was apparent, there was not a statistically significant correlation between aggrecan and Sox9 mRNA expression (Fig. 22C, Spearman r = 0.47, p=0.073). There was no correlation observed between Sox9 and collagen type II (Fig. 22D, Spearman r = -0.055, ns).
Figure 22. Correlation coefficient analysis for patterns of gene expressions in advanced versus minimal OA cartilage.
(A) Statistically significant positive correlation was observed for Bcl-2 and Aggrecan expression. Spearman’s r = 0.827; p = 0.002, directional. (B) Statistically significant positive correlation was observed for Bcl-2 and Sox9 expression. Spearman’s r = 0.691; p = 0.013, directional. (C) A trend was observed for Sox9 and aggrecan correlated expression, but it was not statistically significant. Spearman’s r = 0.473; p = 0.073, directional. (D) No correlation was observed between collagen type II and Sox9. Spearman’s r = -0.055, ns.
DISCUSSION

The initial hypothesis under study in this chapter was directed at establishing that tissue sampled from minimal and advanced OA cartilage on a patient-matched basis would show distinct differences in cellular and matrix characteristics. The samples were obtained based on strict criteria at the gross level: Advanced tissue was located within 1 cm of overt lesions and minimal tissue was obtained from cartilage with no obvious surface lesions (Fig. 17). These samples were analyzed at the histological level using endpoints that relate to the pathogenesis of OA including loss of proteoglycan (Poole et al., 1996; Gebhard et al., 2003), distribution of chondrocytes (Mankin et al., 1971; Tetlow et al., 2001), structural changes such as fibrillation and fissures (Hollander et al., 1995; Gebhard et al., 2003), and cellularity (Brandt, 2001). All endpoints, with the exception of cell density, were found to differ significantly between minimal and advanced OA cartilage, thus confirming our initial hypothesis (Fig. 19).

Since this cartilage is obtained from a joint that is clearly involved in a disease process we assume that the minimal OA cartilage is not exactly comparable to normal cartilage. Future studies will be directed at comparing the minimal OA cartilage with age-matched non-OA cartilage obtained from arthroplasty following surgical removal of chondrosarcomas or hip fracture. However, the strength of the model described in this report lies in the fact that it utilizes minimal and advanced OA cartilage from the same patient, thus controlling for possible differences due to age and genetic variations. Further, all samples were processed for RNA isolation or placed in fixative within 3 hours after isolation from the patient. There are few reported studies which utilize this type of approach in human OA (Kim et al., 2000; Squires et al., 2003; Bock et al., 2001). Bock et al. (2001) reported that the transcription and translation of mRNA for both decorin and biglycan were up-regulated in the maximum loading area of late-
stage OA, compared with adjacent areas in order to compensate for the general loss of proteoglycan associated with this stage of the disease (Bock et al., 2001). A separate study compared lesional and non-lesional areas using post-mortem material, and showed significantly less glycosaminoglycan (GAG) content in the lesional area (Squires et al., 2003). These findings from both these studies are consistent with both the metachromatic scoring data (Fig. 19C) and GAG content (Fig. 20A) reported herein showing a loss of chondroitin sulfate proteoglycan from the advanced OA cartilage in our study. Further studies utilizing models of patient-matched minimal and advanced OA cartilage may help define pathogenic mechanisms operating during OA disease progression.

It is important to compare a model of patient-matched minimal and advanced OA cartilage with other approaches that have been reported in the literature. There have been several recent reports comparing OA tissue to normal tissue. Some examples of cartilage sources reported in these studies include: 1) OA tissue obtained at joint arthroplasty with normal tissue obtained from young donors (Martin et al., 2001; Pullig et al., 2000a; Aigner et al., 2003; Gebhard et al., 2003), 2) OA and normal tissue obtained from different joints (Steck et al., 2002; Martin et al., 2001); and 3) OA tissue obtained from joint arthroplasty with normal tissue obtained from cadavers (Gebhard et al., 2003; Aigner et al., 2003; Cs-Szabo et al., 1997). These studies have reported both consistent and conflicting results with regard to several important endpoints related to the pathogenesis of OA. For example, Martin et al (Martin et al., 2001) compared normal cartilage obtained from femoral neck fracture repair or foot amputations (ankle cartilage) with OA cartilage obtained from various joints at arthroplasty, and found that the ratio of collagen II to collagen I, and aggrecan to versican were higher in control cartilage, suggesting a loss of differentiated phenotype in chondrocytes associated with OA cartilage. However, Gebhard et al. found no evidence for a shift toward a more undifferentiated phenotype in mRNA
expressions of different collagen types from samples based on a model comparing OA cartilage obtained at the time of joint arthroplasty with control cartilage obtained at autopsy within 24-48 hours after death (Gebhard et al., 2003). These differences may relate to the source of the normal cartilage, either the fact that different joints were used or may result from post-mortem changes. In comparing results reported from the different models, the issues of age and genetic background of the patient, post-mortem delay and source of tissue are likely important with no single model being perfect in all aspects.

After characterizing the differences in minimal and advanced human OA cartilage at the cellular and tissue level, we next examined patterns of expression of specific genes. The study focused on matrix genes important for the functioning of articular cartilage (Sandell et al., 2001; Roughley, 2001; Poole et al., 2001), regulatory molecules involved in the control of chondrocyte gene expression and viability (Kinkel et al., 2003), as well as genes coding for specific MMPs potentially important in OA progression (Sandell et al., 2001; Mort et al., 2001; Nagase et al., 2003). We observed a significant decline in the steady-state level of aggrecan and link protein mRNA in advanced OA compared to minimal OA (Fig. 21A). This overall pattern of decreased mRNA coding for aggrecan in the advanced OA cartilage is consistent with both the level of loss of metachromatic staining observed with histology (Fig. 19C) and the decreased GAG content found with the biochemical studies (Fig. 19A). The observed down regulation of aggrecan mRNA in advanced OA cartilage seems, at first, contradictory to reports of up-regulation of collagen type II, aggrecan, link protein and other proteoglycan mRNAs that have been reported in comparisons of normal and OA cartilage (Cs-Szabo et al., 1997; Gebhard et al., 2003; Aigner et al., 2003; Aigner et al., 1999). However, it is likely that that the overall expression of aggrecan may be elevated in OA cartilage compared to normal although advanced OA cartilage may still have less expression compared to minimally involved cartilage. This is supported by studies
using the cruciate ligament transection model for OA in dogs (Matyas et al., 2002). In this study, both aggrecan and collagen type IIB mRNA expression was decreased at 39 weeks after cruciate ligament transection as compared to 10 weeks. The finding suggests that there was down-regulation of expression with advancing disease, even though there was an overall up-regulation of aggrecan and collagen type IIB mRNA at both 10 weeks and 39 weeks in osteoarthritic cartilage compared with normal cartilage.

In contrast to the consistent down-regulation of aggrecan mRNA, some patients studied showed a decrease in the collagen type II mRNA level in the advanced OA cartilage, while the remainder showed an increase or no change (Fig. 21A). There are several possible explanations for this variability in collagen II mRNA expression observed in this study. First, it is known that the mRNA coding for collagen II is expressed as at least two isoforms (Aigner et al., 1999). The primers used in this study would amplify both isoforms, namely type IIA and IIB. There are reports that during late stages of OA there is increased overall expression of type II collagen and the type IIA isoform is preferentially increased (Gebhard et al., 2003; Aigner et al., 1999; Nelson et al., 1998; Nelson et al., 1998). In addition, the expression of collagen II has been shown to be variable where collagen type II and Sox9 protein expression was lost in areas of degeneration, but still expressed in areas adjacent to defects based on immnuohistochemistry (Salminen et al., 2001). Therefore, variable patterns of collagen type II mRNA expression might depend on the severity of OA, the size of the lesions, and/or sampling location. This more complicated pattern of collagen gene expression is likely related to the finding that, in contrast to GAG content, biochemical analysis showed no difference in total hydroxyproline content in advanced OA compared to minimal OA cartilage. Future studies can be directed at a more precise determination of collagen expression using collagen type specific primers for quantitative real time PCR and immunohistochemistry.
A striking finding in this study was that the steady state level of mRNA coding for osteopontin mRNA was significantly increased in advanced compared to minimal OA cartilage in virtually all of the patients studied (Fig. 21A). During endochondral ossification, chondrocytes differentiate into hypertrophic chondrocytes and express osteopontin, which is not normally expressed by mature human articular chondrocytes (Gerstenfeld et al., 1996; Nakase et al., 1994; Sommer et al., 1996; Binette et al., 1998). Furthermore, several reports have confirmed that osteopontin expression is increased, both at the mRNA and protein level, in human OA (Pullig et al., 2000a; Martin et al., 2001). The expression level of osteopontin in these studies depended on the severity of the disease, with expression limited to the deep zone of cartilage in early stages of OA and the strongest expression observed in chondrocyte clusters at late stages of OA (Pullig et al., 2000a). Our data along with the published studies suggest that OA progression in humans involves up-regulation of a protein that is associated with terminal differentiation of chondrocytes. It will be important to determine if the mRNA and protein expression of other markers of terminal differentiation, such as collagen X (Von der et al., 1992), are up-regulated in the advanced OA cartilage as well.

MMPs have an important, but still incompletely understood role in OA disease progression. The expression levels of specific MMPs likely vary during the different stages of OA and in different locations of OA cartilage (Shlopov et al., 1997; Freemont et al., 1997; Tetlow et al., 2001). Here we focused on MMP3, 9 and 13 mRNA expressions in minimal and advanced OA cartilage (Fig. 21C). The expression levels of all 3 MMPs were found to be generally decreased in advanced OA cartilage compared to minimal tissue. However, a subset of patients showed increased expression of these 3 MMPs in advanced OA cartilage compared to minimal OA cartilage. Shlopov et al. reported increased MMP1 and MMP13 mRNA expression in lesional OA cartilage compared to non-lesional OA cartilage obtained from the same knee
(Shlopopov et al., 1997). It has also been reported that MMP3 and MMP9 mRNAs are expressed in the deep zone of cartilage and have different patterns of expression related to different disease stages (Freemont et al., 1997). Tetlow et al. have shown that these particular MMPs are expressed more in the superficial zone than the deep zone (Tetlow et al., 2001). Therefore, the variability in expression of MMP3, 9 and 13 observed in the present study may be related to patient-to-patient variation in the ratio of deep and superficial zone cartilage that was present. The lack of demonstration of a consistent change in expression of mRNA coding for different MMPs is likely due to the heterogeneity of chondrocytes that exists even in a well-defined sample set. It would be possible in future studies to subdivide the samples even further with regard to superficial versus deep zones. The data presented in this report provides a clear foundation for using the sensitive technique of real-time PCR to support this type of analysis.

Finally, we examined the relative expression of Sox9 and Bcl-2 in minimal versus advanced OA cartilage. Bcl-2 expression was decreased in seven of the patients examined with the remainder showing increased or similar expression levels in advanced versus minimal OA cartilage (Fig. 21B). Bcl-2 protein expression has been detected in OA cartilage, with lesional areas showing higher expression than non-lesional by immunohistochemistry (Kim et al., 2000). There is also a report that Bcl-2 expression is decreased in OA cartilage compared with normal cartilage (Erlacher et al., 1995). The more variable pattern of expression observed in our study might result from heterogeneity of chondrocytes within either the minimal or advanced OA cartilage. With regard to Sox9, there was a more consistent pattern of down-regulation of mRNA expression in advanced OA cartilage compared with minimal OA cartilage (Fig. 21B). Variable patterns of Sox9 expression have been reported in different studies. For example, in degenerative lesions, chondrocytes forming clusters show strong immunostaining for Sox9, even though other chondrocytes did not show Sox9 positive staining (Salminen et al., 2001). On the other hand,
another study reported that both the protein and mRNA levels of Sox9 were decreased in OA cartilage compared with either normal cartilage or cartilage in early stages of OA (Aigner et al., 2003). Therefore, additional studies are required to determine how these important regulatory molecules are operating in the pathogenesis of progressive OA.

Previously, we reported data suggesting that Bcl-2 modulates chondrocyte matrix gene expression, especially aggrecan, through a mechanism involving Sox9 in isolated chondrocytes (Kinkel et al., 2003; Feng et al., 1999). Therefore, in this study we looked for correlated patterns of expression of matrix genes, Sox9, and Bcl-2 across all patients (Fig. 22). A statistically significant positive correlation was found between the expression levels of Bcl-2 and aggrecan mRNA (Fig. 22A). A statistically significant positive correlation was also observed between the levels of mRNA coding for Sox9 and Bcl-2 (Fig. 22B). In addition, there was no significant correlation found for the expression levels of mRNA coding for Sox9 and aggrecan, although a clear trend was apparent (Fig. 22C). It was interesting that no correlation was observed between the mRNA levels coding for Sox9 and Collagen type II (Fig. 22D) as was reported previously (Aigner et al., 2003). This finding probably reflects the complexity of regulation of the type II collagen gene in vivo. In addition to positive regulatory factors, the collagen type II gene is also regulated by inhibitory transcription factors such as delta EF-1 (Murray et al., 2000). Also, regulation by Sox 9 appears to be dependent on other co-regulatory proteins such as Sox5 and Sox6 (Lefebvre et al., 2001). These interactions may also help explain the lack of a strong correlation between expression levels of Sox9 and aggrecan as well. However, overall, these data support a hypothesis that Bcl-2 may also play a role in regulating the differentiated phenotype of the articular chondrocyte in vivo as well as in vitro. The finding in chapter two that Bcl-2 regulates Sox9-dependent matrix protein expression via MEK-ERK1/2 signaling pathway in in vitro implicates the involvement of MEK-ERK1/2 signaling pathway in osteoarthritis as
discussed in discussion section of chapter four. The results of in vitro studies can then be further confirmed using similar approaches and sample sets as described in this report.

This data further defines an approach for analyzing gene expression patterns of chondrocytes associated with different stages of OA using an intrajoint comparison model. The advantage of this approach is that it offers better control over confounding variables such as age, genetic background, differences in clinical management, and artifacts due to specimen collection since the major comparisons are made between cartilage from the same individual. The findings suggest that the phenotypic change in chondrocytes with advanced disease involve down-regulation of steady-state mRNA coding for aggrecan and link protein and up-regulation of osteopontin gene expression. This hypothesis will need further confirmation using variations of the existing model in order to determine the influence, if any, of the specific joint compartment on patterns of gene expression. For example, studies are now planned to compare cartilage obtained from patients with OA associated with valgus alignment to our current data set. It has been clearly demonstrated that valgus alignment results in a pattern of disease progression with more severe lateral compartment involvement as compared to the medial compartment (Cahue et al., 2004).

The further analysis of human OA using intrajoint comparison and including age-matched non-OA samples will continue to refine our understanding of the pathogenic mechanisms that underlay this important disease.
CHAPTER FOUR
GENERAL DISCUSSION AND FUTURE DIRECTIONS

Discussion

Bcl-2, an anti-apoptotic protein, functions as a regulator of cell behavior in several cell types beyond apoptosis. Additionally, the loss of Bcl-2 expression correlated with an altered chondrocyte phenotype such as decreased cartilage matrix protein expression was found in previous studies (Feng et al., 1998; Feng et al., 1999; Kinkel et al., 2003). The following two hypotheses, therefore, were examined 1) Bcl-2 regulates Sox9-dependent chondrocyte matrix gene expression through specific signaling pathway, and 2) The expression pattern of Bcl-2 and chondrocyte specific proteins will vary in chondrocytes associated with different stages of human osteoarthritis.

A role for Bcl-2 in maintaining the chondrocyte phenotype was presented in chapter two. The antisense Bcl-2 cell lines have some apoptosis based on DNA ladder formation (Feng et al., 1998; Feng et al., 1999) and are very sensitive to apoptotic stimuli. However, these cells do remain viable and have a fibroblastic phenotype increasing decreased chondrocyte matrix gene expression and increased type I collagen gene expression. In the present study, a Bcl-2 siRNA vector was used to see the direct effect of suppression of Bcl-2 on chondrocyte matrix gene expression, since stable transfection may cause secondary effects. The Sox9-dependent collagen type II promoter activity was decreased in cells transfected with Bcl-2 siRNA for 48h. This downregulation was not recovered with the caspase inhibitor, ZVAD, which blocks apoptosis. This data indicates that Bcl-2 regulates matrix protein transcription independent of apoptosis. Moreover, the Sox9-dependent collagen type II transcription depends on the level of Bcl-2 in chondrocytes as demonstrated in figure 7. The downregulation of Bcl-2 and Sox9-dependent
chondrocyte matrix protein transcriptions was also found in wild type chondrocytes exposed to serum withdrawal that is known to induce apoptosis and decrease Bcl-2 expression in chondrocytes as well as in many other cell types (Kinkel et al., 2003; Yagi et al., 2005a). Chondrocytes are known to lose response to trophic factors, so the serum withdrawal in wild type IRC mimics the condition seen in chondrocytes during aging (Martin et al., 2002). The constitutive Bcl-2 expression in IRC cells and primary chondrocytes maintained the Sox9-dependent matrix transcription under serum withdrawal. Taken together, the decreased Bcl-2 alters chondrocyte ability to avoid apoptosis as well as ability to maintain matrix production.

The finding that Bcl-2 regulates matrix protein has implication to studies of aging. The number of chondrocytes are decreased during aging due to apoptosis (Adams et al., 1998a; Livne, 1994). Chondrocytes also lose the ability to repair the damaged tissue and respond to stimuli such as epidermal growth factor and insulin growth factor (Carrington, 2005). We have reported previously that Bcl-2 declines in articular cartilage during aging (Kinkel et al., 2004). In addition to Bcl-2 expression, matrix protein expression is also decreased during aging (Bolton et al., 1999; Buckwalter et al., 1994; Martin et al., 2002; Verbruggen et al., 2000). Thus, Bcl-2 might play a role as a regulator of matrix protein expression in chondrocytes during aging.

Chapter Three presented in vivo data supporting the hypothesis that Sox9-dependent matrix expression is regulated by Bcl-2. There was correlation between the expression of mRNA coding for Bcl-2 and matrix proteins in advanced OA relative to minimal OA. The downregulation of chondrocyte matrix protein mRNAs is somewhat surprising, since the well-accepted idea is that chondrocyte matrix proteins are increased in osteoarthritis compared with normal cartilage. However, this theory is not always consistent among many studies, as discussed in the introduction of chapter three. Moreover, the source of cartilage is different in the present study compared to other reported studies. In the present study, the relative mRNA
expression was calculated by creating ratio of minimal and advanced OA cartilage. The minimal OA cartilage is not considered as intact-normal cartilage, since the minimal OA cartilage is in the same knee where the advanced OA cartilage was obtained. In contrast, other reported studies compare the mRNA level in normal cartilage with osteoarthritic cartilage taken from other individuals. This compares heterogeneous tissue that is found in osteoarthritis. Thus, our data do not contradict previously reported studies showing increased matrix expression in osteoarthritis.

By combining both our data and previous data, I suggest that overall matrix protein expression is increased in osteoarthritis compared with normal cartilage. However, the induction of matrix proteins might be highest in minimal OA cartilage and then this induction declines in advanced OA cartilage, but the matrix protein expression is still higher than aging normal cartilage. To address this issue, the matrix protein mRNA expression in normal cartilage should be determined and relative expression in normal, minimal OA, and advanced OA cartilage should be compared.

Chapter Two also demonstrated that Bcl-2 regulates Sox9-dependent chondrocyte matrix gene expression through MEK-ERK1/2 pathway. The inhibition of MEK activity overcomes the loss of Bcl-2 effect on chondrocytes by reversing the cell morphology and increasing chondrocyte matrix proteins as indicated in Figure 13 and 14. Thus Bcl-2 is upstream of MEK-ERK1/2 cascade that regulates Sox9 transcription and activity (Fig. 23). The mechanism of the ERK1/2 effect on Sox9 is still unknown. Sox9 should be translocated into the nucleus in order to act as transcription factor. However, the regulation of Sox9 localization has not yet been identified (Fig.23). I have looked at the level of phospho-ERK1/2 in cytoplasm and nucleus in wild type IRC cells treated with serum withdrawal (data not shown). Serum withdrawal resulted in the accumulation of phospho-ERK1/2 in the nucleus and reduction in the cytoplasm. The localization of Sox9 and Phospho-ERK1/2 should be verified to understand if ERK1/2 phosphorylation influences Sox9 in the cytoplasm or the nucleus. The possible mechanisms
linking ERK1/2 and Sox9 are discussed in the future direction section. Interestingly, aggrecan transcription did not respond to 25µM MEK inhibitor in antisense Bcl-2 cell lines, even though collagen type II and Sox9 transcriptions were up-regulated with the inhibitor. In contrast to antisense Bcl-2 cell lines, wild type chondrocytes induced aggrecan transcription as well as Sox9 and collagen type II transcription in the presence of MEK inhibitor. Other signaling pathways, PKC\(\alpha\) and NF\(\kappa\)B, are not involved in the Bcl-2/Sox9 signaling pathway even though these signals are known to regulate Sox9-dependent matrix expression (Fig. 23). Additionally, the MEK-ERK1/2 cascade limits the ability of the mature chondrocyte to express matrix proteins as described in Figure 12 and 13. PKC\(\alpha\) and ERK1/2 activation is inversely regulated in chondrogenesis (Yoon et al., 2002). However, the phosphorylation of ERK1/2 was not affected by PKC phosphorylation.

Thus, MEK-ERK1/2 is a negative pathway for the stable chondrocyte phenotype, which is maintained by Bcl-2. The model, Bcl-2/MEK-ERK/Sox9, presented here has implication not only in mature chondrocytes but also in osteoarthritis. The Bcl-2 and chondrocyte matrix protein mRNA expression are coordinately decreased in advanced OA cartilage compared with minimal OA cartilage as shown in chapter three (Yagi et al., 2005b). This indicates that Bcl-2 is also involved in the expression of chondrocyte matrix proteins in osteoarthritis as well as stable mature chondrocytes. An experimental animal model shows that the inhibition of MEK block the progression of osteoarthritis (Pelletier et al., 2003). Additionally, osteoarthritic cartilage showed a large number of phosph-ERK1/2 positive cells. In contrast, a very low number of cells in normal cartilage was stained with a phospho-ERK1/2 antibody. The authors suggested that the increased ERK1/2 phosphorylation in osteoarthritis leads to the expression of MMP-1 that degrades collagen. Thus, the inhibition of MEK-ERK1/2 cascade blocks the matrix degradation by MMP-1. From studies presented here, activation of MEK-ERK1/2 pathway also induces the
downregulation of Sox9-dependent matrix protein expression. Taken together, the suppression of Bcl-2 increases phosphorylation of ERK1/2 that decreases the expression of chondrocyte matrix protein expression and increases MMP-1 expression in osteoarthritis. Therefore, MEK inhibitor might be a possible therapy for osteoarthritis.

In addition, the mechanical regulation of ERK1/2 signaling in articular cartilage has also been suggested (Fanning et al., 2003). This study revealed a rapid induction of ERK1/2 phosphorylation and a sustained level of signaling in response to compression and IGF-I. The authors suggest that IGF rapidly distributes to cartilage to induce the phosphorylation of ERK1/2 in response to compression, since IGF-I is the predominant anabolic growth factor in synovial fluid. Static compressive forces also can down-regulate chondrocyte matrix protein expression (Kim et al., 1996; Ragan et al., 1999; Sah et al., 1989). By linking these studies with our present work, we hypothesized that the activation of MEK-ERK1/2 cascade might decrease chondrocyte matrix protein in response to compression.

There is no study describing the relationship between phosphorylation of ERK1/2 and aging in chondrocytes. However, it is reasonable to consider the possible induction of phospho-ERK1/2 with aging, since we have reported that Bcl-2 expression declines with aging in articular cartilage (Kinkel et al., 2004) and others reported that chondrocyte matrix protein expression is also decreased during aging (Bolton et al., 1999; Buckwalter et al., 1994; Carrington, 2005; Livne, 1994; Martin et al., 2002).

Two other studies showed an involvement of Raf-1 or ERK1/2 signaling in Bcl-2 function beyond regulating apoptosis. Trisciuoglio et al., reported that Bcl-2 up-regulates urokinase plasminogen activator receptor (uPAR) expression by increasing Sp1 binding activity via activation with ERK1/2 signaling pathway in cancer cells. However, the authors did not find any mechanism that links Bcl-2 and ERK signaling and suggested a possible involvement of
protein kinase C, calcium and other molecules without specific hypothesis. Haughn et al. (2003), reported that the interaction of Raf-1 and Bcl-2 might regulate the lineage choice of hematopoetic progenitor cells. The possible underlying mechanism was that the BH4 domain of Bcl-2 binds to the catalytic domain of Raf-1, but this suggested mechanism is not the complete story. The interaction of Bcl-2 and Raf-1 is discussed in the next section.
Figure 23. Schematic summary of proposed mechanisms of Bcl-2 function in regulating matrix gene expression. Note that the activation or inhibition of Raf-1 can be regulated through many pathways. This regulation is dependent on cell type and stimuli. The present study identified the involvement of the MEK-ERK1/2 cascade but not PKC and NFκB. In the normal condition, Bcl-2 inhibits the activation of the MEK-ERK1/2 cascade and maintain Sox9-dependent matrix expression. The loss of Bcl-2 activates the pathway and inhibits the Sox9-dependent matrix gene expression. The possible underlying mechanism by which Bcl-2 regulates the MEK-ERK1/2 cascade is discussed in the next section. The transcription of aggrecan can be regulated by Sox9 and other transcription factors or signaling pathway regulated by the MEK-ERK1/2 pathway.
Future directions

The work presented here could lead to several future studies. The present and previous work in our laboratory confirmed that Bcl-2 regulates matrix expression in addition to apoptosis. Bcl-2 influences the MEK-ERK1/2 pathway to regulate Sox9 activity. There is additional work required to understand the mechanism by which Bcl-2 influences the MEK-ERK1/2 pathway and how ERK1/2 influences Sox9. In addition, the role of MEK-ERK1/2 should be examined further in osteoarthritis. The future directions suggested here are only a few of many possibilities.

1. The mechanism linking Bcl-2 and MEK1/2.

The studies presented here suggest that Bcl-2 involves the MEK-ERK1/2 signaling pathway to regulate Sox9 mRNA expression and activity (Fig. 23). The loss of Bcl-2 activates the signaling pathway followed by the decreased Sox9 mRNA expression and activity. Future work should be directed at identifying the molecules or pathway mediating the link Bcl-2 and MEK1/2. MEK1/2 is phosphorylated (activated) by Raf-1 and active MEK1/2 phosphorylates ERK1/2 which has more fifty substrates. There are three isoforms of Raf; Raf-1, A-Raf, and B-Raf, but all three Raf isoforms share MEK as the only commonly accepted downstream substrates (Kolch, 2000). Since a MEK inhibitor blocks the activity of MEK to phosphorylate ERK1/2, the suppression of Bcl-2 must activate MEK1/2 at a downstream target. However, there is no other upstream target for MEK1/2 except Raf-1. Several Raf-associated proteins are suggested including Bcl-2 and chaperon proteins such as Bag-1, a Bcl-2 binding protein (Kolch, 2000). For example, the interaction of Bcl-2 and Raf-1 allows Raf-1 kinase to enhance the anti-apoptotic activity of Bcl-2 by phosphorylating Bad (Wang et al., 1996). There is one report showed that Bcl-2 associates with Raf-1 to dictate the fate of differentiation in hematopoietic progenitor cells (Haughn et al., 2003). This study suggested that the interaction between Raf-1 and BH4 domain
of Bcl-2 influences the cell decision to become myeloid. The exchange of the BH4 domain of Bcl-2 to the BH4 domain of Bcl-X₇ made cells become erythroid by increasing the Raf-1 level.

Bcl-X₇ belongs to Bcl-2 family that has anti-apoptotic function. However, the interaction of Raf-1 and the BH4 domain of Bcl-X₇ has not been determined. The increased Bcl-X₇ relative to Bcl-2 level might interfere with the interaction of Raf-1 and Bcl-2. The precise mechanism underlying the Raf-1 and Bcl-2 interaction is still unknown. Moreover, the hematopoietic differentiation study did not show either activation or inactivation of Raf-1-MEK-ERK1/2 pathway. The decreased Raf-1 level does not have to result in the inactivation of the MEK-ERK1/2 pathway, since Raf-1 is a kinase. Moreover, Raf-1 is considered to be expressed ubiquitously. Further information will be needed to confirm this model.

The studies presented here revealed that the loss of Bcl-2 activates the MEK kinase and, likely, the Raf-1 kinase. Due to the decreased Bcl-2 level, it is unlikely to have direct interaction of Bcl-2 and Raf-1 in Bcl-2 deficient cells. The Bcl-2 deficient chondrocytes might compensate the loss of Bcl-2 by increasing the level of Bcl-X₇ for survival. The increased Bcl-X₇ might somehow activate Raf-1 kinase and then activates MEK-ERK1/2 pathway in chondrocytes as suggested in the Bcl-2 and Bcl-X₇ regulation of hematopoietic progenitor differentiation (Haughn et al., 2003).

Bag-1 can be another candidate to mediate Bcl-2 and Raf-1 activation. The interactions between Bcl-2 and Bag-1, and Raf-1 and Bag-1 have been suggested (Cutress et al., 2002). Bag-1 is also suggested to be a mediator of the interaction between Raf-1 and Bcl-2 to redirect Raf-1 to the mitochondrial membrane where Bad is inactivated by Raf-1, but there is no definitive proof (Kolch, 2000). Bcl-2 can bind with Bag-1 in normal conditions (Takayama et al., 1995). Bag-1 tends to bind Raf-1 and activates the MEK-ERK1/2 cascade in proliferation (Song et al., 2001; Wang et al., 1996). In stress conditions, Bag-1 tends to bind with HSP70 heat shock protein that
assists HSP70 function, which helps proteins to obtain functional confirmation (Song et al., 2001). It is possible that Bag-1 likely interacts with Bcl-2 in normal conditions and this might interfere with the interaction of Bag-1 and Raf-1. However, Bag-1 might favor an interaction with Raf-1 in Bcl-2 deficient cells leading to activation of Raf-1 kinase. The interaction of Raf-1 and Bag-1 might activate Raf-1/MEK-ERK1/2 pathway to influence Sox9. Another possibility is that the formation of a Bcl-2/Bag-1/Raf-1 complex might be mediated by other molecules when Bcl-2 is suppressed. Thus, in this way, Bcl-2 might be protected from proteolysis for future cell survival.

2. The mechanism linking between ERK1/2 and Sox9.

In order to establish the mechanism linking between ERK1/2 and Sox9, it is first necessary to determine if the protein level of Sox9 is down-regulated in Bcl-2 deficient cells. The Sox9 protein level is most likely decreased, since Sox9 mRNA expression and Sox9-dependent collagen type II promoter activity are suppressed in antisense Bcl-2 cells. ERK1/2 might simply target the transcription of Sox9. However, the precise mechanism regulating Sox9 transcription is still unknown. ERK1/2 may increase suppressor proteins or decrease activator proteins binding to regulatory regions of the Sox9 gene to decrease Sox9 transcription. This hypothesis can be examined by generating many constructs containing mutations of possible transcriptional regulatory regions.

ERK1/2 might not target Sox9 transcription directly, but instead regulate known ERK1/2 nuclear targets, such as Elk, signal transducers and activators of transcription1/3 (STAT1/3), activation protein-1 (AP-1) and serum response factor (SRF). The inhibition of MEK activity enhances the expression of SRF and AP-1 as well as Sox9 and collagen type II expression (Seghatoleslami et al., 2003). Thus, SRF and AP-1 can be targets of ERK1/2 and decreased SRF
and AP-1 might suppress Sox9 transcription. In general, the Elk, and STAT1/3 transcription factors can be phosphorylated by ERK1/2. The phospho-Elk or STAT1/3 might be suppressors of Sox9 transcription. However, STAT1/3 is not likely involved in the pathway, since STAT1/3 was not found in wild type, antisense Bcl-2 and sense Bcl-2 IRC cell lines with or without serum withdrawal (data not shown).

There are additional studies that should be carried out to see the other possible mechanisms how ERK1/2 regulates Sox9 besides the Sox9 transcription step. The localization of phosphor-ERK1/2 protein should be identified as discussed above. From my study, phosphor-ERK1/2 likely translocates to the nucleus to regulate Sox9. If phospho-ERK1/2 stays in the cytoplasm, the protein might inhibit the translocation of Sox9 from the cytoplasm to the nucleus. The identification of Sox9 localization in Bcl-2 deficient cells should also be determined.

The other possibility is the phosphorylation of Sox9 by ERK1/2. It is suggested that Sox9 can be phosphorylated by PKA and this enhances its binding and transcriptional activity (Huang et al., 2000). However, the phosphorylation of Sox9 is not well characterized yet. Thus, it is possible that ERK1/2 can phosphorylate Sox9 and lead the suppression of Sox9 activity.

3. The role of Bcl-2 and MEK-ERK1/2 signaling in osteoarthritis.

The Bcl-2 and chondrocyte matrix protein expression was decreased in advanced OA compared with minimal OA cartilage. The loss of Bcl-2 activates MEK-ERK1/2 cascade to suppress Sox9 in vitro. Therefore, it is reasonable to hypothesize that MEK-ERK1/2 signaling is also activated in advanced OA cartilage compared with minimal OA cartilage. This hypothesis can be addressed by analyzing the localization of phospho-ERK1/2 in minimal and advanced OA cartilage using immunostaining. Pelletier et al. (2003), confirmed that a larger number of
phosph-ERK1/2 positive cells are found in osteoarthritic cartilage compared with normal cartilage in an animal model.

Since our laboratory has the ability to isolate and culture human OA cartilage, the role of Bcl-2 in isolated advanced OA cartilage should be studied. The chondrocytes in advanced OA cartilage have decreased Bcl-2 and chondrocyte matrix protein expression compared with minimal OA cartilage as described in chapter three. The infection of sense Bcl-2 adenovirus might bring back the expression of collagen type II, aggrecan, and Sox9 transcription in chondrocytes from advanced OA cartilage. Moreover, the induction of Bcl-2 in advanced OA cartilage with Bcl-2 adenovirus may decrease phosphorylation of ERK1/2, since phosphorylation of ERK1/2 might be increased in advanced OA. Additionally, these experiments can be expanded using normal aging cartilage and minimal OA cartilage.
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