Bcl-2 Positively Regulates Sox9-dependent Chondrocyte Gene Expression
By Suppressing the MEK-ERK1/2 Signaling Pathway
Rieko Yagi, Denise McBurney, and Walter E. Horton Jr.
Department of Anatomy, Northeastern Ohio Universities College of Medicine,
4209 State Route 44 Rootstown, Ohio 44272

Running title: Bcl-2 modulates chondrocyte gene expression via ERK1/2
Address correspondence to: Walter E. Horton Jr., Department of Anatomy, Northeastern Ohio Universities College of Medicine, 4209 State Route 44 Rootstown, Ohio 44272, Tel. 330-325-6290; Fax. 330-325-5913; E-Mail: wehj@neoucom.edu

Bcl-2 is an anti-apoptotic protein that has recently been shown to regulate other cellular functions. We previously reported that Bcl-2 regulates chondrocyte matrix gene expression, independent of its anti-apoptotic function. Here, we further investigate this novel function of Bcl-2, and examine three intracellular signaling pathways likely to be associated with this function. The present study demonstrates that the activity of Sox9, a master transcription factor that regulates the gene expression of chondrocyte matrix proteins, is suppressed by Bcl-2 siRNA in the presence of caspase inhibitors. This effect was attenuated by prior exposure of chondrocytes to an adenoviral vector expressing sense Bcl-2. In addition, the down-regulation of Bcl-2, Sox9 and chondrocyte-specific gene expression by serum withdrawal in primary chondrocytes was reversed by expressing Bcl-2. Inhibition of the PKCα and NFκB pathways had no effect on the maintenance of Sox9-dependent gene expression by Bcl-2. In contrast, whereas the MEK-ERK1/2 pathway negatively regulated the differentiated phenotype in wild type chondrocytes, inhibition of this pathway reversed the loss of differentiation markers and fibroblastic phenotype in Bcl-2-deficient chondrocytes. In conclusion, the present study identifies a specific signaling pathway namely, MEK-ERK1/2, that is down stream of Bcl-2 in the regulation of Sox9-dependent chondrocyte gene expression and phenotype.

Bcl-2 is a well known anti-apoptotic protein (1). Accumulating evidence suggests that Bcl-2 regulates other cellular behavior, in addition to or independent of apoptosis (2-11). For example, the growth and regeneration of retinal or trigeminal axons is promoted by Bcl-2 (3;6). Bcl-2 is also necessary for the early maturational change in embryonic sensory neurons (10), and dictates the differentiation of hematopoetic progenitor cells (5;9). Furthermore, the terminal differentiation of keratinocytes is inhibited by Bcl-2 (4), 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 (11). Bcl-2 overexpression in melanoma cells under hypoxia conditions modulates vascular endothelial growth factor expression (2;7). 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 (8). 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.

Chondrocytes, the only resident cells in cartilage, synthesize matrix proteins including aggrecan, collagen type II and link protein that are important for tissue function (12). The transcription factor Sox9 binds to regulatory regions of genes coding for these matrix proteins as well as collagen type XI and cartilage derived retinoic acid-sensitive protein (CD-RAP) (13-16). Sox9 is also required for chondrocyte differentiation and chondrocyte proliferation (17;18).

During development, chondrocytes differentiate from mesenchymal cells and are responsible for longitudinal growth of long bones (19). Chondrocytes in the prehypertrophic zone of the growth plate express Bcl-2 along with cartilage matrix proteins and Sox9. (19-23). However, in the hypertrophic zone where chondrocytes undergo terminal differentiation and start to express collagen type X and bone related matrix proteins, there is decreased expression of Bcl-2
and cartilage matrix proteins as well as loss of Sox9 expression (24-27). In vivo, suppression of Bcl-2 expression leads to accelerated terminal differentiation of chondrocytes and shortened long bones and Bcl-2 is downstream of parathyroid hormone related peptide (PTHrP) (28).

Previously we reported that Bcl-2 functions as a regulator of cartilage matrix protein expression in chondrocytes (29-31). The suppression of Bcl-2 in chondrocytes results in decreased aggrecan expression even when the apoptotic pathway is blocked by inhibition of caspase activity (30). Additionally, aggrecan, collagen type II, link protein and Sox9 mRNA expression is significantly decreased in chondrocytes expressing a low level of Bcl-2 (30;31). However, collagen type I mRNA expression is increased suggesting that Bcl-2 may be involved in regulating the phenotype of chondrocytes (31). Furthermore, chondrocytes constitutively expressing Bcl-2 are protected from downregulation of aggrecan, collagen type II and Sox9 following serum withdrawal (30;31). 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.

Here we tested three possible pathways that might 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 (32-34). NFκB inhibits Sox9 activity, destabilizes Sox9 mRNA, and decreases collagen type II expression with TNFα treatment (35;36). The ERK1/2 signaling pathway is known to negatively regulate collagen type II, aggrecan and Sox9 mRNA in mesenchymal cells prior to chondrocyte differentiation (37;38). Conversely, the ERK pathway has been shown to positively regulate Sox9 mRNA and activity during chondrogenesis induced by fibroblast growth factor (39). 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 (40). The findings presented here support a 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.

MATERIALS AND 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 (41). Cells were transfected with plasmids containing sense or antisense Bcl-2 coding sequences and selected in G418 as described previously (29;30). The cells were plated at a density of 1 × 10^6 in T25 flasks for protein isolation or 3.85 × 10^5 in 6-well pates for RNA extraction and were cultured at 37°C with 5% CO₂ in Ham’s F-12 medium.

Adenoviral Vectors and Infection in Chondrocytes - The adenovirus vectors were generated with 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 (29). 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 Adeno-X Rapid Titer Kit (BD Clontech). The infection efficiency of adenovirus, as determined by β-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 ×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 phosphorylated 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 immunofluorescence staining, the primary Bcl-2 antibody and anti-rabbit FITC secondary antibody were purchased from Santa Cruz Biotechnologies. The MEK1/2 inhibitor, U0126 (Cell Signaling), was used at concentrations of 20 or 25µM depending on experiments. The PKCα inhibitors, Go6983 (42) and GF109203X (43) (Sigma) were used at concentrations of 1µM and 5µM which have been shown to suppress PKCα activity (40;44). 50µM ZVAD (Alexis) was used to inhibit general caspase activities.

**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 Na3VO4, 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 (14;45), (generous gift from Dr. V. Lefebvre, Cleveland Clinic Foundation) or an NfkB reporter construct (Stratagene) was co-transfected with a β-galacosidase reporter vector as an internal control of transfection efficiency. The dominant negative IκB expression vector (Stratagene), or Bcl-2 or control siRNA vector (IMGENEX) were also co-transfected with the Sox9 construct and β-galacosidase reporter vector. All transfections were performed with Lipofectamine2000 (Invitrogen) mixed with OptiMEM (Gibco). Cells were exposed to DNA-Lipofectamine complex in 10% FBS Ham’s F-12 without antibiotics for 4h and then cultured overnight in normal growth media containing 10% FBS. After recovery, the cells were exposed to inhibitors or serum withdrawal as indicated. The cells were then lysed with Reporter Lysis Buffer (Promega). β-galacosidase 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 × 10^4/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 (46). The Alcian Blue bound to matrix proteoglycan was
extracted with 4M guanidine hydrochloride and measured at 600nm (32).

**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 were designed and examined for primer efficiency as described previously (31). Briefly, primers were designed using Primer Express software (Applied Biosystems) and primer efficiency was performed with a standard curve of 60, 6, and 0.6ng 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_T Method (User Bulletin #2, Applied Biosystems, and reference, (31)).

**RESULTS**

The level of Bcl-2 protein modulates Sox9-dependent chondrocyte matrix gene expression - We previously demonstrated that 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 (29-31). Here we examine the effects of direct down-regulation of Bcl-2 using siRNA on Sox9-dependent collagen type II promoter activity. IRC cells were transfected with a plasmid vector expressing siRNA for Bcl-2 along with the Sox9 reporter construct. Immunocytochemistry was used to demonstrate no change in the Bcl-2 level in IRC chondrocytes transfected with control siRNA containing random DNA sequences (Fig. 1A), while there was an obvious decrease in the percentage of chondrocytes expressing Bcl-2 following transfection with the Bcl-2 siRNA (Fig. 1B).

The Sox9-dependent reporter construct contains four copies of the Sox9 binding site upstream from a minimal collagen II promoter (14:45). 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 compared with cells transfected with control siRNA (Fig. 2A). This suppression of Sox9 activity was observed even in the presence of 50µM ZVAD, a general caspase inhibitor that blocks full apoptosis in IRC cells (30) compared with control siRNA transfected cells treated with 50µM ZVAD (Fig. 2B). 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 increasing basal expression of 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 in the presence of 10% FBS. Cells infected with LacZ virus and transfected with Bcl-2 siRNA showed a 50% down-regulation of Sox9-dependent promoter activity compared with control siRNA (Fig. 3). However, if the cells were first treated with Bcl-2 adenovirus in order to increase the basal level of Bcl-2 there was no decrease in Sox9 dependent promoter activity following transfection with Bcl-2 siRNA (Fig. 3).

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 (31). 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 (29).
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 under serum withdrawal treatment compared with 10% FBS treated wild type cells (Fig. 4). However, there was no loss of Sox9-dependent promoter activity following serum withdrawal in the two cell lines with constitutive Bcl-2 expression (Fig. 4).

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, Sox9 and Bcl-2 mRNA was determined (Fig. 5). Adeno-LacZ infected cells showed decreased steady state levels of mRNA coding for all four transcripts 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. 5).

The NFkB 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 NFkB (35). In addition, NFkB has been shown to be involved in the suppression of chondrogenesis and destabilization of Sox9 mRNA (36). Therefore, we considered the possibility that NFkB might be down stream of Bcl-2 in regulating Sox9-dependent chondrocyte gene expression. A vector containing four repeats of an NFkB 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 NFkB activity in wild type IRC cells (Fig. 6A) even though Sox9-dependent promoter activity was significantly decreased under the same condition (Fig. 4). The Sense Bcl-2 cell lines also did not show any effect of serum withdrawal on NFkB activity. However, the level of NFkB activity in IRC cell lines with constitutive expression of Bcl-2 was three-fold higher than in wild type cells (Fig. 6A). This suggested a possible role for high basal activity of NFkB in the protective effect of Bcl-2 on Sox9-dependent promoter activity. Therefore, we used a dominant negative IxB expression vector, co-transfected with the Sox9 reporter construct and a β-galactosidase expression construct to test this hypothesis. The dominant negative IxB expression vector effectively blocked NFkB activity in both wild type and sense Bcl-2 IRC cell lines (data not shown). However, there was no change in Sox9-dependent promoter activity under these conditions in either wild type or sense Bcl-2 IRC cell lines cultured in 10% FBS (Fig. 6B). These data do not support a role for NFkB in the pathway by which Bcl-2 regulates chondrocyte gene expression.

Bcl-2 regulation of chondrocyte phenotype is not dependent on PKCa - PKCa has been shown to promote chondrogenesis by activating collagen type II expression, and to block the loss of the differentiated phenotype of chondrocytes (33;40). The level of phosphorylated PKCa is decreased in wild type IRC cells cultured in 0% FBS compared to cells cultured in 10% FBS (Fig. 7, Lanes 1-2). In contrast, sense Bcl-2 cell lines showed a high level of phosphorylated PKCa and this level was maintained following serum withdrawal (Fig. 7, Lanes 3-6). Further, two independent antisense Bcl-2 IRC cell lines showed decreased PKCa phosphorylation compared with wild type cells (data not shown). The data suggests that PKCa 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 transfected with the Sox9-dependent promoter reporter construct and treated with two different PKCa inhibitors, Go6983 and GF109203X. Both inhibitors resulted in decreased Sox9 dependent promoter activity in wild type IRC cells compared to cells treated with vehicle (Fig. 8A). 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. 8B). These results suggest that PKCα signaling is not required for the Bcl-2 dependent pathway that maintains Sox9 activity.

**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 (37;38;47-49). 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. 9A). Serum withdrawal for 24 hours resulted in strongly increased phosphorylation of ERK1/2, but the total ERK1/2 level was unaffected (Fig. 9A). 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. 9B). Note that the time of exposure of the blot to film in Fig. 8B, was relatively long compared to that shown in Fig. 8A. The inhibition of phosphorylated ERK1/2 resulted in a strong up-regulation of the steady state level of mRNA coding for aggrecan, collagen II, and Sox9 (Fig. 9C) as well as an increase in the Sox9-dependent promoter activity (Fig. 9D). 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 (46). 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. 10).

**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 (31). The level of phospho-ERK1/2 but not total ERK1/2 is up-regulated in two different antisense Bcl-2 cell lines consistent with a role in the negative regulation of the differentiated chondrocyte phenotype (Fig. 11A). Inhibition of MEK1/2 using the U0126 inhibitor resulted in a suppression of phospho-ERK1/2 in the AS-8 line (Fig. 11B, Lanes 1-2).

The inhibition of the MEK-ERK pathway resulted in a reversal of the fibroblastic phenotype observed for the antisense Bcl-2 line at 24h (Fig. 12A-C). An accumulation of cartilage matrix proteoglycan as measured by Alcian Blue staining was evident by 48h (Fig. 12D-F). The same increase in Alcian Blue positive matrix was observed in another independent antisense Bcl-2 cell line treated with 25µM U0126 (data not shown). In addition, the steady state level of mRNA transcripts for collagen II and Sox9 were up-regulated four-fold with the MEK inhibitor, U0126 (Fig. 13A). However, aggrecan mRNA expression was not increased at 24h, but was increased three-four fold after 48h of exposure to the MEK inhibitor.

Finally, the relationship between phospho-ERK1/2 levels and Sox9-dependent promoter activity was examined following knock down of Bcl-2 expression with siRNA (Fig. 13B). 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. 13B). 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.
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 (29-31). 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 suggesting a role for Bcl-2, other than that of regulating the mitochondrial pathway of apoptosis (6,10,16,18,19,23,28,32,33,48). 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 (5). Others also report that Bcl-2 inhibits terminal differentiation of keratinocytes by decreasing expression of keratin10/11, a differentiation marker (4). 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 (11).

Our first goal in the present study was to provide a foundation for identifying possible signaling pathways related to Bcl-2 regulation of differentiated chondrocyte phenotype. 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 (31). 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 (Fig. 2B). Since siRNA can have non-specific effects (50;51), and to show the specificity of our observation, we used an alternative technique and demonstrated that increasing the baseline level of Bcl-2 by transfection of Bcl-2 siRNA (Fig. 3). Herein, we also report that independent clonal IRC chondrocyte lines constitutively expressing Bcl-2 are protected from loss of Sox9 activity following serum withdrawal (Fig. 4). 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 (Fig. 5). 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 NF\textkappaB, PKC\alpha, and MEK-ERK1/2. NF\textkappaB has been shown to either promote or block apoptosis depending on the stimulus (52;53). In the case of chondrocytes, NF\textkappaB is known to inhibit Sox9 activity, destabilize Sox9 mRNA, and decrease collagen type II expression with TNF\alpha treatment (35;36). In the present study, we observed that NF\textkappaB activity was not changed with serum withdrawal in wild type IRC cells, even though Sox9-dependent promoter activity was decreased (Fig. 4 and Fig. 6A). However, the basal level of NF\textkappaB activity was higher in IRC chondrocyte lines expressing increased and constitutive levels of Bcl-2 compared to wild type IRC chondrocytes (Fig. 6A). These observations led us to hypothesize that NF\textkappaB may be involved in maintaining Sox9 activity and chondrocyte specific gene expression. However, this hypothesis could not be supported because, although a dominant negative I\textkappaB expression vector transfected into chondrocytes reduced NF\textkappaB activity, there was no effect on Sox9-dependent promoter activity in either wild type or IRC-Bcl-2 lines (Fig. 6B). This suggests that NF\textkappaB is not required for the Bcl-2 mediated Sox9 dependent promoter activity. In fact, studies have implicated NF\textkappaB signaling pathway as a negative regulator of Sox9 activity and collagen type II mRNA expression following TNF\alpha treatment (35;36). It is possible that the elevated Bcl-2 in the IRC lines is able to overcome any potential negative regulation by NF\textkappaB.
Because it has been reported that PKCα is a positive regulator of chondrogenesis (32;40;47), and that the PKCα signaling pathway is also involved in the maintenance of the differentiated chondrocyte phenotype (40), we hypothesized that PKCα was possibly downstream of Bcl-2. This hypothesis was supported by the finding that the phosphor-PKCα level declined with serum withdrawal in wild type IRC chondrocytes but not in IRC cell lines with constitutive expression of Bcl-2 (Fig. 7). 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. 8A) and proteoglycan synthesis (data not shown) in wild type IRC. Previous work using a mesenchymal cell model, showed that the level of PKCα increased during chondrogenesis (40). 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 cell 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 (Fig. 8B). This significant finding suggests that Bcl-2 expression, above a critical level, overcomes the need for PKCα in 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 (54).

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, aggregan, and Sox9 (32;37;49). 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 (38;39;55). 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 (Fig. 9A). Interestingly, the present study shows that inhibition of the low level of ERK1/2 in the wild type cells actually up-regulates the steady state level of mRNA coding for aggregan, collagen II, and Sox9, as well as Sox9 activity (Fig. 9C and 9D), similar to what has been reported with increased expression of Bcl-2 (31). It was also striking that the already differentiated IRC cells could be induced to accumulate even more Alcian blue positive extracellular matrix (Fig. 10). However, it is likely that much of the matrix secretion was lost into the culture medium (37), 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 (40). However, U0126 used in the current study is a more specific MEK inhibitor (56). Collectively, our data suggests that endogeneous phosphorylation of ERK1/2 serves as a limiting regulator of Sox9-dependent cartilage matrix protein expression.

The observed inverse 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, ERK1/2 was highly phosphorylated (Fig. 11). It has been shown previously that these antisense Bcl-2 lines have lost the differentiated chondrocyte phenotype (29-31). In the present study, we used a MEK inhibitor to up-regulate the steady state level of mRNA coding for aggregan, collagen II, and Sox9, as well as to stimulate Sox9 activity in the antisense Bcl-2 lines (Fig. 13A and 13B). In addition, we demonstrated that the fibroblastic phenotype of the antisense Bcl-2 IRC chondrocytes was reversed with inhibition of MEK by 24h and that the cells accumulated abundant Alcian Blue positive extracellular matrix by 48h (Fig. 12). Collectively, these findings indicate that the suppressive action of MEK-ERK
signaling pathway on Sox9-dependent gene expression in chondrocytes is inhibited by Bcl-2. 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 (11). 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 (57). 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. We have reported that the expression level of Bcl-2 declines with aging in articular chondrocytes in mice (58). It has been reported that aging of cartilage is associated with both an increase in the number of apoptotic chondrocytes and a decrease in the response of chondrocytes to growth factors as well as decreased chondrocyte matrix protein expression (59-62). We have recently reported that there is a correlation between the expression of Bcl-2, Sox9 and aggrecan in chondrocytes located in human osteoarthritic articular cartilage (63). Furthermore, inhibition of the MEK-ERK pathway can prevent the progression of osteoarthritis by maintaining the chondrocyte phenotype (64). Thus, clearly, there is *in vivo* significance with respect to the signaling pathway by which Bcl-2 regulates the chondrocyte phenotype.

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 (11). 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.

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**Figure Legends**

**Fig. 1.** 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.

**Fig. 2.** 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. After transfection, cells were treated with either 0.2% Me2SO or 50µM ZVAD, a potent caspase inhibitor for 48h in the presence of 10% FBS. The relative promoter activity was defined as promoter activity in cells transfected with Bcl-2 siRNA divided by the activity in cells transfected with control siRNA. Sox9 dependent promoter activity was suppressed by Bcl-2 siRNA to 30% of control siRNA in the absence (A) or presence (B) of ZVAD.
**FIG. 3.** The 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 in the presence of 10% FBS with Bcl-2 or control siRNA, the Sox9-dependent reporter construct and the β-galactosidase expression plasmid. After transfection, cells were cultured for 48h in the presence of 10% FBS. 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. Statistical significance was determined by Wilcoxon signed-rank test; *** P< 0.001, compared to control.

**FIG. 4.** The constitutive expression of Bcl-2 protects against down-regulation of Sox9-dependent promoter activity following serum withdrawal. The Sox9 dependent reporter construct and β-galactosidase plasmid were transfected into wild type IRC chondrocytes or two independent IRC lines constitutively expressing Bcl-2 (S-1 and S-2) in the presence of 10% FBS. After transfection, all cells were serum deprived for 24 and 48hr. The relative promoter activity was defined as promoter activity in cells cultured in 0% serum divided by activity in cells cultured in 10% serum. The promoter activity in cells cultured with 10% serum was assigned a value of 1.0 in all cases. 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 Wilcoxon signed-rank test. The asterisk indicates a statistical significant decreased in promoter activity in wild type cells cultured in 0% serum compared to control (10% serum) * P< 0.05.

**FIG. 5.** 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 (col2), aggrecan (Agg), Sox9 and Bcl-2 mRNA. The relative mRNA expression was defined as mRNA expression in cells cultured in 0% serum divided by mRNA expression in cells cultured in 10% serum. The mRNA expression in cells cultured with 10% serum was assigned a value of 1.0 in all cases. 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.

**FIG. 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 in the presence of 10% FBS with NFκB reporter construct and β-galactosidase plasmid and then exposed to serum withdrawal for 24hr. 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 treated with 10% FBS (*** P< 0.001). (B) Wild type IRC chondrocytes were transiently transfected in the presence of 10% FBS with a dominant negative IκB expression vector and Sox9-dependent reporter construct. After transfection, cells were cultured for 48h in the presence of 10% FBS. No effect of the dominant negative IκB on Sox9 dependent promoter activity was observed. The relative promoter activities are mean ± S.D. of three independent experiments. Statistical analysis on data presented in (A) and (B) was determined by Wilcoxon signed-rank test.

**FIG. 7.** The phosphorylation of PKCα in wild type and sense Bcl-2 cell lines with serum withdrawal. Chondrocytes were incubated for 24hr 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. Lane1, wild type IRC in 10% FBS; lane 2, wild type IRC in 0% FBS; lane 3, S-1 IRC (sense Bcl-2) in 10% FBS; lane 4, S-1 IRC in 0% FBS; lane 5, S-2 (sense Bcl-2) IRC in 10% FBS; lane 6, S-2 IRC in 0% FBS. Serum withdrawal suppressed the phosphorylation of PKCα in wild type IRC (** P< 0.01), 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 Wilcoxon signed-rank test with each culture condition compared back to wild type IRC cells cultured in 10% serum (value was set at 1.0).

**Fig. 8. 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 1µM or 5µM Go6983 (Go) or GF109203X (GF), or 0.1% or 0.5% Me2SO vehicle alone for 24h in the presence of 10% FBS. The Sox9 activity was depressed following treatment with two different inhibitors in wild type IRC cells (** P< 0.01; * P< 0.05). (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 analyzed by Wilcoxon signed-rank test comparing the relative promoter activity in control cells (DMSO treated) with cells exposed to the two different inhibitors.

**Fig. 9. The effect of endogenous phosphorylated ERK1/2 in wild type IRC cells.** (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. (C) Wild type IRC chondrocytes were treated with 0.2% Me2SO or 20µM U0126 treatment for 24h, and relative mRNA was determined by quantitative real time PCR. The relative mRNA expression was defined as mRNA expression in cells cultured in 25µM U0126 divided by mRNA expression in cells cultured in vehicle. The mRNA coding for aggrecan (Agg), collagen type II (Col2), and Sox9 were up-regulated 2-3 fold following inhibition of phospho-ERK1/2. (D) 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 up-regulation 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.

**Fig. 10. 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 in the presence of 10%FBS. (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.

**Fig. 11. The phosphorylation of ERK1/2 in antisense Bcl-2 IRC cell lines.** (A) The two independent antisense Bcl-2 cell lines showed high level of phosphorylated ERK1/2 compared with wild type IRC cells, whereas total ERK1/2 level was similar. (B) Antisense Bcl-2 cell line was treated with 0.25% Me2SO (Lane1) or 25µM U0126 (Lane 2) in presence of 10%FBS. The MEK inhibitor decreased the level of phosphorylated ERK1/2 in the antisense Bcl-2 IRC line. The data shown represent typical results obtained from three independent experiments. Two different antisense IRC cell lines showed similar results.
Fig. 12. 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 24h (A, B, C) and 48h (D, E, F) incubation in medium containing, no treatment, 0.25% Me₂SO (vehicle) or 25µM U0126 in the presence of 10% FBS. The fibroblastic phenotype in the antisense Bcl-2 cells without any treatment (A, D) was maintained with 0.25% Me₂SO (B, E). U0126 reversed the fibroblastic phenotype to a chondrocytic phenotype at 24h without significant proteoglycan accumulation (C). U0126 dramatically increased Alcian Blue staining in the pericellular matrix at 48h (F). The data shown represent typical results obtained from three independent experiments.

Fig. 13. 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 U0126, or 0.25% Me₂SO as a control for 24h. The relative mRNA expression was defined as mRNA expression in cells cultured in 25µM U0126 divided by mRNA expression in cells cultured in the vehicle. The mRNA expression in cells cultured with 10% FBS was assigned a value of 1.0 in all cases. The mRNA level of collagen type II (Col2), and Sox9 was increased with the MEK inhibitor in antisense Bcl-2 cell line. Aggrecan (agg) mRNA expression was not induced at 24h, but increased at 48h (data not shown). The relative mRNA expression is the mean ± S.D. of four independent experiments. Statistical significance was determined by Wilcoxon signed-rank test. The asterisk indicates a statistically significant increased in mRNA expression in cells cultured in U0126 compared to vehicle, *P<0.05, **P<0.01. (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 U0126, or 0.25% Me₂SO. The relative promoter activity was defined as promoter activity in cells transfected with Bcl-2 divided by activity in cells transfected with control siRNA. Sox9-dependent promoter activity was decreased in siRNA transfected chondrocytes treated with vehicle control but was maintained by treatment with the MEK inhibitor. Representative data from two different cell lines showed equivalent data. The mean ± S.D. was calculated from four independent experiments.
Figure 1

A

B
Figure 2

A

Bcl-2 siRNA / Control siRNA

Relative Sox9 Dependent Promoter Activity

Control siRNA  Bcl-2 siRNA

0.0  0.5  1.0

***

B

50uM ZVAD

Bcl-2 siRNA / Control siRNA

Relative Sox9 Dependent Promoter Activity

Control siRNA  Bcl-2 siRNA

0.0  0.5  1.0

***
Figure 3
Figure 4

![Bar chart showing relative Sox9 dependent promoter activity at 24hr and 48hr with different cell lines and FBS concentrations.](chart1)

Figure 5

![Bar chart showing relative mRNA expression of Col2, Agg, Sox9, and Bcl-2 at 0% and 10% FBS with different adenovirus constructs.](chart2)
Figure 6

A

B

Relative Sox9 Dependent Promoter Activity

WT S-1 S-2

10% Serum
0% Serum

Control
IkB / Control Vector
Figure 7

Relative Phospho-PKC Protein Level
Phospho-PKC / Actin

Figure 8

A

Relative Promoter Activity
PKC Inhibitors / Vehicle

B

Relative Sox9 Dependent Promoter Activity
PKC Inhibitors / Vehicle

Vehicle
Go
GF
Figure 9

A

B

ERK1/2-P

ERK1/2

10% FBS

0% FBS

Vehicle

U0126

C

D

Relative mRNA Expression

Relative Sox9-dependent Promoter Activity

U0126 / Vehicle

0.0

0.5

1.0

1.5

2.0

2.5

24h

48h

Agg

Col2

Sox9

Vehicle

U0126
Figure 10
Figure 11

A

| WT | AS-3 | AS-8 |
|----|------|------|
| ERK1/2-P |  |  |
| ERK1/2 |  |  |

B

| 1 | 2 |
|---|---|
| ERK-P |  |
| ERK1/2 |  |
Figure 12

A: 24 hr
B: 25uM U0126
C: Non-treated Vehicle
D: 48 hr
E: Vehicle
F: 25uM U0126
Figure 13

A

### Relative mRNA Expression

|        | Agg | Col2 | Sox9 |
|--------|-----|------|------|
| U0126  | 1.0 | *2.5* | **3.0** |
| Vehicle| 1.0 | 1.2  | 1.5  |

B

### Relative Sox9 Dependent Promoter Activity

|        | DMSO | U0126 |
|--------|------|-------|
| Bcl-2  | 1.0  | *1.5* |
| Control siRNA | 1.0 | 1.2 |

* indicates p < 0.05, ** indicates p < 0.01.