Regulation of SOX9 mRNA in Human Articular Chondrocytes Involving p38 MAPK Activation and mRNA Stabilization*

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Human articular chondrocytes rapidly lose their phenotype in monolayer culture. Recently we have shown that overexpression of the transcription factor SOX9 greatly enhanced re-expression of the phenotype in three-dimensional aggregate cultures. Here we show that endogenous SOX9 mRNA can be rapidly up-regulated in subcultured human articular chondrocytes if grown in alginate, in monolayer with cytochalasin D, or with specific inhibition of the RhoA effector kinases ROCK1 and -2, which all prevent actin stress fiber formation. Disruption of actin stress fibers using any of these redifferentiation stimuli also supported the superinduction of SOX9 by cycloheximide. The superinduction was blocked by inhibitors of the p38 MAPK signaling pathway and involved the stabilization of SOX9 mRNA. Furthermore stimulation of chondrocyte p38 MAPK activity with interleukin-1β resulted in increased levels of SOX9 mRNA, and this was again dependent on the absence of actin stress fibers in the cells. In this study of chondrocyte redifferentiation we have provided further evidence of the early involvement of SOX9 and have discovered a novel post-transcriptional regulatory mechanism activated by p38 MAPK, which stabilized SOX9 mRNA.

Articular chondrocytes are the only cell type found within articular cartilage and are responsible for the formation and maintenance of the specialized extracellular matrix of the tissue. Isolation of these cells and culture in monolayer dramatically alters their differentiation state. They change from a phenotype expressing COL2A1 (encoding collagen type II α1) to one expressing COL1A1 (encoding collagen type I α1) and switch from a rounded to a fibroblastic morphology. During early passages in monolayer culture, chondrocytes are able to re-express their COL2A1 phenotype if placed in a culture that promotes a round cell shape (1) or in a high density cell aggregate culture (2). The transcription factor SOX9, which plays a major role in the regulation of COL2A1 gene expression, has been shown to be involved in this redifferentiation process (3, 4). SOX9 expression is progressively lost during monolayer culture of human articular chondrocytes, and work by our laboratory has shown that retroviral expression of SOX9, even after extensive passage in monolayer, can reinstate the potential of the cells to respond to cell rounding and cell aggregate culture (5–7).

Regulation of SOX9 gene expression has been extensively studied in developmental systems, yet precise mechanisms that control it are elusive. Study of the immediate 5′ promoter region of SOX9 has demonstrated binding and activation by the CCAAT binding factor (8). Additionally in vitro analysis of chondrocytes and chondrocytic cells has demonstrated regulation of SOX9 mRNA levels by fibroblast growth factor 2 (9), interleukin-1β (IL-1β) (10) and dexamethasone (11). However, analysis of promoter activity is complicated by the presence of several regulatory regions extending at least a megabase both upstream and downstream of the SOX9 locus (12–14) that are thought to confer complex, tissue-specific expression patterns.

The organization of the chondrocyte cytoskeleton is clearly of importance in the full expression of chondrocyte phenotype, and this may be regulated by many interacting signaling pathways. The p38 MAPK is a stress-activated protein kinase related to JNK and is able to transduce a variety of stress and physiological signals into transcriptional regulation (15). Such stress signals include the protein synthesis inhibitor cycloheximide, which can lead to superinduction of genes by p38 MAPK-dependent mechanisms (16, 17). In chondrocytes, cytokines such as IL-1β and tumor necrosis factor α signal through the p38 MAPK pathway (18), and this contributes to their metabolic and catabolic effects (19, 20). Furthermore p38 MAPK signaling has been implicated in the maturation of chondrocytes in the endochondral growth plate during development (21). This activation of p38 MAPK can be opposed by the activity of the RhoA small GTPase, which prevents chondrocyte maturation (22). RhoA is an important regulator of cytoskeletal structure and focal adhesion maturation (23). Its activity promotes the formation of actin stress fibers through downstream effector kinases such as ROCK1 and -2. These act by both increasing tension within the cell through direct phosphorylation of myosin II regulatory light chain (24) and controlling downstream events that inhibit the activity of the actin depoly-

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2 The abbreviations used are: IL, interleukin; ARE, AU-rich element; CHX, cycloheximide; CyD, cytochalasin D; ENaC, epithelial Na+ channel; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase-2; MKK, mitogen-activated protein kinase kinase; ROCK, RhoA effector kinase; UTR, untranslated region; P, passage; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase.
merizing protein coflin (25). Recent studies have shown that prevention of actin stress fiber formation achieved through inhibition of RhoA signaling leads to increased levels of SOX9 mRNA and protein in chondrocytic cells (26).

**EXPERIMENTAL PROCEDURES**

*Cell Isolation and Culture*—Human articular cartilage was obtained with fully informed consent and ethical approval within 18 h of surgery from total knee arthroplasties (6, 7). Chondrocytes were isolated following sequential trypsin/collagenase digestion and cultured at 37 °C in monolayer in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, and 10% fetal bovine serum (all from Cambrex, Wokingham, UK). Experiments were carried out in 25-mm-diameter polystyrene wells unless otherwise stated. Cycloheximide (5 μM) was added to monolayer cultures to prevent de novo protein synthesis. Alginate saline the cells were mounted under a glass coverslip using 100 μd of 5% alginate as 1:2 split ratio. Alginate bead cultures were established as described previously (6). Specific inhibitors were dissolved in dimethyl sulfoxide unless otherwise stated. Cycloheximide (5 μg/ml in ethanol), Y27632 ROCK inhibitor (10 μM in water), SP600125 JNK inhibitor (25 μM), and actinomycin D (1 μM) were all obtained from Calbiochem. Cytochalasin D (1 μM) and SB202190 p38 MAPK inhibitor (20 μM) were obtained from Sigma. Final concentrations of carrier solvents were equal under all experimental conditions. Cells treated with IL-1β (R&D Systems, Abingdon, UK) were in serum-free Dulbecco's modified Eagle's medium.

*Examination of Cytoskeleton in Monolayer and Alginate Chondrocyte Cultures*—Monolayer cultures grown on 8-chamber LabTek II glass slides (Nalge Nunc International, Hereford, UK) were fixed for 10 min at 4 °C with formaldehyde, permeabilized in Tris-buffered saline + 0.1% Triton X-100 for 10 min and then blocked in Tris-buffered saline + 0.1% bovine serum albumin for 1 h. F-actin was stained with rhodamine-conjugated phalloidin (50 μl of 5 units/ml) (Invitrogen) for 30 min. After counterstaining of cell nuclei for 1 min with 300 nM 4',6-diamidino-2-phenylindole and then washing in Tris-buffered saline the cells were mounted under a glass coverslip using Vectashield (Vector Laboratories, Peterborough, UK). Alginate beads were blocked, permeabilized, and stained as described above but with the reagents supplemented with 1 mM CaCl₂ to prevent depolymerization of the alginate. After the final wash step, the stained beads were placed within microcavity slides, soaked with Vectashield, and gently crushed under a glass coverslip. Fluorescence localization was analyzed using a Leica SP2 confocal microscope.

*Gene Expression Analysis*—Real-time PCR was used to examine expression of SOX9 in the chondrocyte cultures. Total RNA was prepared from cell monolayers in 12-well culture plates using 1 ml of TRI reagent (Sigma) per well. Alginate beads were crushed in TRI reagent, and total RNA was extracted into an aqueous phase after the addition of chloroform. This aqueous phase was then passed through a GenElute RNA purification kit (Sigma). cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase and primed with random hexamer oligonucleotides (Promega, Southampton, UK) in a 25-μl reaction. Amplification by PCR was carried out in 25-μl reaction volumes on an MJ Research Opticon 2 using reagents from a real-time PCR probe core kit with gene-specific primers and TaqMan probes (all from Eurogentec, Seraing, Belgium). GAPDH primers and probe have been described previously (27). Primers and probe for SOX9 were designed using Applied Biosystems Primer Express software and had the following sequences: Forward 5'-3', CCCCCACAGATCGCTACAG; Reverse 5'-3', GAGTTCG-GTCCGGTTGA; and Probe 5'-3', CGGCCATCACCCGCTACAGTA. The final concentration for all primers was 300 nM and for probes was 100 nM. Relative expression levels were normalized to GAPDH and calculated using the 2−ΔΔCt method (28). Prior validation had established that both GAPDH and SOX9 PCRs proceeded with a similar efficiency. For determination of matrix metalloproteinase-13 (MMP-13) gene expression, SYBR Green detection was used, and values were normalized using GAPDH. MMP-13 primers were as follows: Forward 5'-3', TGTTGTGCTTACAAAGCAATGGTT; and Reverse, 5'-3' TGCTCTCATTGACAGACCATGTG. For the mRNA decay experiment, SOX9 mRNA copy number in each sample was calculated using a calibration curve created from known dilutions of the pcDNA3SOX9_UT_FLAG vector, a kind gift from Professor Benoit de Crombrugghe, Houston, TX (3). Copy numbers were then normalized to input RNA concentrations, which were measured on a GeneQuant II spectrophotometer.

*Western Blot Analysis of Cell Extracts*—The culture medium was removed, and the cells were washed with Tris-buffered saline before being extracted in 100 μl of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 0.01% (w/v) bromphenol blue) with the aid of a cell scraper. Samples were heated at 80 °C for 10 min under reducing conditions (50 mM dithiothreitol) and run on Novex 4–12% SDS-PAGE gels (Invitrogen). The separated proteins were then blotted onto Biotrace NT nitrocellulose membranes (Pall Corp., Ann Arbor, MI) and probed with the following antibodies: anti-p38 (catalog number 9212), anti-p38 phospho-Thr180/Tyr182 (catalog number 9211), anti-phospho-MAPKAPK2 (catalog number 3041), anti-c-Jun (catalog number 9165), or anti-phospho-c-Jun Ser73 (catalog number 9164). All antibodies were obtained from Cell Signaling Technologies (Danvers, MA), and the manufacturer’s own probing protocol was used. Primary antibody binding was localized using a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (catalog number 7074, Cell Signaling Technologies) and developed using chemiluminescence (Western Lightning Plus, PerkinElmer Life Sciences).

*Gene Sequence Analysis of SOX9 3′-Untranslated Region (UTR)*—SOX9 3′-UTR sequence was obtained in three ways. First, the sequences of the 3′-UTR of human (accession number NM_003046), mouse (accession number NM_011448), Xenopus (accession number NM_00101606853), and rainbow trout (accession number AF209872) SOX9 mRNA were obtained from the GenBank™ data base. Second, sequences 3′ of the SOX9 coding region were identified for rat and dog (each with 80–90% identity to human sequence) from genomic sequence aligned using the University of California Santa Cruz Genome Browser. Finally BLAST (Basic Local Alignment Search Tool) searches of expressed sequence tag data bases using human SOX9 3′-UTR sequences were able to identify highly conserved (>81–91% similarity) cow (accession numbers CB437176 and
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The loss of SOX9 expression is a major factor causing the change in chondrocyte phenotype in monolayer culture (4). In this study there was a striking decrease in SOX9 gene expression in the human articular chondrocytes in monolayer culture (Fig. 1A). Cells at the end of passage 2 (P2) had 10-fold lower levels of SOX9 mRNA than cells freshly isolated from tissue (P0). Many redifferentiation strategies for monolayer expanded articular chondrocytes have been described (1, 2, 29–31). Typically these have included inducing a rounded cell morphology, such as in alginate or cell aggregate culture, and these strategies have been shown to lead to increased expression of target genes of SOX9 including cartilage-specific extracellular matrix proteins. We investigated the early response of chondrocytes to alginate bead culture, which induced cell rounding. The change in cytoskeletal organization was assessed by determining the actin distribution with rhodamine-conjugated phalloidin (Fig. 1B). In monolayer the human articular chondrocytes showed characteristic F-actin stress fibers across the cell. In contrast confocal images of articular chondrocytes after 72 h in alginate revealed a cortical distribution of F-actin and an absence of stress fibers. Analysis of SOX9 gene expression using real time PCR (Fig. 1C) showed that in alginate there was a 5-fold increase in SOX9 mRNA at 72 h. From this experiment, it was clear that cell rounding and/or disruption of actin stress fibers led to an increase in SOX9 gene expression during the early stages of articular chondrocyte redifferentiation in alginate. To determine whether cell rounding was an important aspect controlling the increase in SOX9 we determined SOX9 expression in human articular chondrocytes in monolayer cultured (a) with cytochalasin D (CytD), which prevents actin polymerization, or (b) with Y27632, an inhibitor of the RhoA effector kinases ROCK1 and -2, which are important in the signaling processes that control the formation of actin stress fibers. Therefore both agents inhibit the formation of actin stress fibers but by separate mechanisms. Imaging of the cells after 72 h (Fig. 2) showed that untreated controls contained large stress fiber bundles. CytD treatment (0.1 μM) caused a slight retraction of the cells, but actin stress fibers were still present. However, at 1 μM CytD many of the cells were fully retracted into a rounded shape, and at 10 μM CytoD all cells had a round shape, and no actin stress fibers were visible. Specific inhibition of RhoA signaling using Y27632 had little effect at 1 μM, but at 10 μM the cells assumed a thin elongated morphology with an absence of actin stress fibers. At 100 μM Y27632 in addition to the absence of actin stress fibers there was also cell rounding. Both CytD and Y27632 caused significant increases in SOX9 gene expression at their two higher concentrations, and this corresponded with a complete loss of actin stress fibers in each case. The results with Y27632 suggested that the rounding up of the cell was less important than the loss of actin stress fibers. It seems likely therefore that prevention of actin stress fiber formation plays an important role in controlling SOX9 gene expression levels during the early stages of redifferentiation of cultured mature chondrocytes.

To determine whether new protein synthesis was required for the induction of SOX9 in alginate culture, we tested the effects of the protein synthesis inhibitor cycloheximide (CHX) (Fig. 3A). In control cultures the time course showed that SOX9 was increased almost 5-fold as early as 24 h after the formation of the alginate beads. The addition of CHX had a surprising inductive effect on SOX9 mRNA, which rose almost 20 fold higher after 48 h. In contrast with the chondrocytes in mono-

CO896800) and horse (accession numbers CX603491 and CX604580) sequences. All known and putative SOX9 3′-UTRs were aligned with human SOX9 3′-UTR using ClustalW, and conserved AU-rich elements (AUUUA) were identified.

RESULTS

FIGURE 1. Modulation of SOX9 expression in monolayer expanded human articular chondrocytes. A, real time PCR analysis of cDNA from human articular chondrocytes freshly isolated from tissue (P0) or after one or two passages in monolayer culture (P1 and P2); data are average values from three different donors. *, p < 0.05 versus P0, unpaired t test. B, fluorescence micrographs showing rhodamine-conjugated phalloidin staining of actin in chondrocytes grown as monolayers or in alginate beads. Scale bars, 10 μm. C, SOX9 gene expression determined by real time PCR analysis of P2 human articular chondrocytes grown as monolayer cultures or for 72 h in alginate beads. Results are representative of duplicate experiments carried out with cells from different donors (n = 3). *, significant increase (p < 0.05) compared with monolayer, unpaired t test.
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layer culture with CHX (Fig. 3B) there was no induction of SOX9 over 24 h. However, if there was additional disruption of actin with CytD, or Y27632, then SOX9 mRNA was increased almost 4-fold by CHX. These results provided evidence of another mechanism for increasing SOX9 mRNA levels in articular chondrocytes that was triggered by CHX but only occurred in the absence of ROCK-dependent actin structures.

To further define the mechanism by which CHX increased SOX9 mRNA, we tested whether it was sensitive to the inhibition of signaling through the stress-activated protein kinase pathways p38 MAPK and JNK, which CHX is known to activate (32, 33). To disrupt stress fibers in monolayer cultures treated with CHX, Y27632 was chosen rather than CytD because of its greater specificity to those actin structures. Preliminary experiments showed that increases in SOX9 mRNA were easily detected at 5 h, and this short time was chosen for further experiments as it reduced the risk of cell toxicity that might be caused by multiple signaling pathway inhibitors over longer treatment. In control cultures analysis of cell extracts revealed higher levels of activated phospho-p38 MAPK in the presence of CHX (Fig. 4A) as well as increased c-Jun phosphorylation, which confirmed that CHX caused both p38 MAPK and JNK activation. The ROCK inhibitor Y27632 increased further the p38 MAPK activation but decreased c-Jun phosphorylation. Inhibitor SB202190 strongly inhibited the CHX-mediated increase of SOX9 mRNA in alginate. However, the experiment also showed no inhibition of the increased expression caused by the alginate culture itself (Fig. 4D). The JNK inhibitor SP600125 had no effect on SOX9 levels in any alginate culture conditions. JNK signaling was therefore not involved. The inhibition of stress-activated protein kinases in these experiments showed that activation of p38 MAPK, but not JNK, was necessary for the up-regulation of SOX9 mRNA in CHX-treated human articular chondrocytes. Additionally the inhibition of actin stress fiber formation was an essential part of the action of p38 MAPK. It was also noted that because neither of these stress-activated protein kinase pathway inhibitors affected the up-regulation of SOX9 caused purely by cell rounding in alginate this was independent of p38 MAPK and JNK activation.

Superinduction of gene expression by CHX can be caused by increased transcription or increased mRNA stability (16, 17). We investigated the stability of SOX9 mRNA in CHX/Y27632-treated monolayer cultures using actinomycin D to inhibit transcription. The stability of SOX9 mRNA was found to be much greater in CHX/Y27632-treated cultures (half-life, 3.1 h) than in controls (half-life, 1.8 h) (Fig. 5). Furthermore co-incubation of cells with CHX/Y27632/SB202190 significantly reduced the SOX9 mRNA stabilization (half-life, 2.4 h) demon-
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The expression of SOX9 appears to be important for the phenotypic stability of chondrocytes and essential for their ability to assemble a cartilage matrix (4, 7). In monolayer culture the expression of SOX9 falls, but some recovery has been shown to be promoted by culture under conditions that favor cell rounding, such as in alginate (31). In this study we investigated some of the mechanisms that regulate SOX9 expression in human articular chondrocytes, which had been isolated and cultured in monolayer. Initial results showed that SOX9 mRNA levels increased quickly when the cells were cultured in alginate. A significant increase in SOX9 mRNA levels was detected in these passaged human chondrocytes in alginate (31). In this study we investigated some of the mechanisms that regulate SOX9 expression in human articular chondrocytes in monolayer culture in a dose-dependent manner after 5 h (Fig. 6A). As evidence of downstream signaling, MMP-13 gene expression was measured using real time PCR and was greatly increased after exposure to IL-1β (Fig. 6B). Treatment of the cells with either IL-1β or Y27632 (10 μM) alone had no effect on SOX9 mRNA levels, but combining IL-1β with Y27632 led to significant increases in SOX9 mRNA at IL-1β concentrations of 1 and 10 ng/ml (Fig. 6C). Inhibition of p38 MAPK with SB203580 resulted in reduced levels of SOX9 mRNA and prevented the IL-1β/Y27632-induced increase in SOX9 expression (Fig. 6C). This result showed that in these passaged human chondrocytes in monolayer culture SOX9 was up-regulated by IL-1β by a p38 MAPK-dependent mechanism just as in the response to CHX.

DISCUSSION

Stratifying that p38 MAPK signaling was involved in the mechanism. A similar analysis of actinomycin D-treated alginate cultures also provided evidence of SOX9 mRNA stabilization in CHX superinduction (data not shown). Therefore, the results suggested that SOX9 mRNA stabilization was likely to be a major cause of the p38 MAPK-mediated increase in SOX9 mRNA induced by CHX/Y72632.

The stability of mRNA has been shown to be greatly influenced by the presence of AU-rich elements (AREs) in their 3′-UTRs. Data base analysis of human SOX9 3′-UTRs revealed eight AUUUA motifs, five of which were conserved in the 3′-UTR of rat, mouse, and dog SOX9 (Table 1). All the conserved motifs were among very well conserved AU-rich sequences (between 73 and 85% AU content of the 40 bp surrounding the central uracil of each motif), and two of them also form part of a defined UUAUUAA(U/A)(U/A) minimal destabilizing sequence (34). From the sequence available we found that the latter motifs and two other motifs were conserved in cow and horse expressed sequence tags. No comparable conserved sequences were found in Xenopus and rainbow trout SOX9 3′-UTRs. The results showed that mammalian SOX9 3′-UTRs contained well conserved elements that could render the mRNA susceptible to 3′-ARE-dependent mRNA stabilization signals.

As IL-1β is an important factor that can regulate the anabolic and catabolic activity of chondrocytes and signals through the p38 MAPK pathway, we investigated whether this physiological activation of p38 MAPK also affected SOX9 expression. This was also of particular interest as IL-1β has been reported to down-regulate SOX9 in chondrocytes (10). IL-1β treatment, under serum-free culture conditions, activated p38 MAPK in human articular chondrocytes in monolayer culture in a dose-dependent manner after 5 h (Fig. 6A). As evidence of downstream signaling, MMP-13 gene expression was measured using real time PCR and was greatly increased after exposure to IL-1β (Fig. 6B). Treatment of the cells with either IL-1β or Y27632 (10 μM) alone had no effect on SOX9 mRNA levels, but combining IL-1β with Y27632 led to significant increases in SOX9 mRNA at IL-1β concentrations of 1 and 10 ng/ml (Fig. 6C). Inhibition of p38 MAPK with SB203580 resulted in reduced levels of SOX9 mRNA and prevented the IL-1β/Y27632-induced increase in SOX9 expression (Fig. 6C). This result showed that in these passaged human chondrocytes in monolayer culture SOX9 was up-regulated by IL-1β by a p38 MAPK-dependent mechanism just as in the response to CHX.
CHX is well characterized as a protein synthesis inhibitor (35), but it has also been long appreciated that it can interact in other cellular processes and "superinduce" gene expression (36). In this study it was shown to superinduce SOX9 in chondrocytes in alginate culture. It also became clear from experiments with monolayer cultures that the CHX superinduction of SOX9 mRNA only occurred under conditions where actin stress fiber formation was prevented, such as by treatment with CytD or Y27632. CHX has been shown to cause the superinduction of IL-6 in a number of cell types (16, 37–39) and of epithelial Na+ channel (α-ENaC) in glucocorticoid-treated MDCK-C7 cells (17). Other genes, including growth arrest and DNA damage-inducible gene, cyclooxygenase-2, nucleophosmin, and CYP1A1, have also been reported to be superinduced by CHX (33, 40–42). Here we also show that in chondrocytes p38 MAPK was required for SOX9 mRNA superinduction by CHX. In addition, the activation of p38 MAPK physiologically, by IL-1β signaling, also induced SOX9 but again only in the absence of actin stress fibers caused by ROCK1/2 inhibition. The dependence of actin stress fiber integrity on p38 MAPK activity has been established in other systems. For instance, smooth muscle cell induction of connective tissue growth factor by anisomycin and of cysteine-rich angiogenic inducer-61 by sphingosine 1-phosphate was shown to be dependent on p38 MAPK activity, and in both cases an intact cytoskeleton was required (43, 44).

The experiments with IL-1β demonstrated that activation of p38 MAPK by an extrinsic signal also supported SOX9 induction. However, there were differences in potency between these stimuli. For example, IL-1β was generally less potent than CHX in inducing SOX9, yet it caused greater p38 MAPK activation. This indicates that CHX may activate additional pathways that cooperate with p38 MAPK in SOX9 induction. In both routes the organization of the actin cytoskeleton clearly had an important requirement in controlling induction. The effect of IL-1β to increase SOX9 mRNA in this study is interesting as IL-1β has previously been shown to down-regulate SOX9. Primary neonatal murine costal chondrocytes showed a 10-fold down-regulation of SOX9 within 5 h of treatment with IL-1β that
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The difference in results is likely to be due to the relative levels of SOX9 expression in the two systems and what signals are sustaining SOX9 expression. The young costal chondrocytes were sourced from a fast growing tissue, and the cells expressed high levels of SOX9 (10). This contrasts with the mature human articular chondrocytes in our study that have already down-regulated SOX9 during monolayer culture. In our experiments SOX9 expression was lower, and IL-1β alone had no effect on SOX9 expression after 5 h. The difference in results suggests that the high SOX9 expression in the murine study is dependent on signals, which are suppressed by IL-1β, whereas in the human chondrocytes, those signals may be already lost during monolayer culture. A lack of inhibition of SOX9 expression has also been reported in another study of human osteoarthritic articular chondrocytes cultured with IL-1β for 3 days (45), and evidence for context-dependent responses during chondrocyte differentiation to Rho/ROCK signaling has been reported (46).

The evidence that SOX9 mRNA stimulation by IL-1β required inhibition of stress fiber formation implies that chondrocyte responses to p38 MAPK activity are dependent on cytoskeletal organization. This may explain why signals are interpreted differently between rounded chondrocytes with cortical actin distribution and flattened fibroblastic chondrocytes with well organized stress fibers. Other evidence of such differences has been reported. For example, induction of cyclooxygenase-2 by IL-1β has been reported to differ between alginate- and monolayer-cultured immortalized human articular chondrocytes (47). It is worth noting that despite the importance of p38 MAPK in SOX9 superinduction neither p38 MAPK nor JNK was involved in SOX9 mRNA up-regulation induced solely by alginate culture. Their exclusion as regulators of alginate induction of articular chondrocytes is compatible with studies that implicated an up-regulation of protein kinase C and a decreased activation of extracellular signal-regulated protein kinase in this process (48). Additionally it is worth noting that the p38 MAPK inhibitor SB202190 reduced control SOX9 levels in the IL-1β experiment (Fig. 6C) suggesting a role for the pathway in maintaining the low levels of SOX9 found in dedifferentiated chondrocyte monolayers.

Superinduction of genes by CHX has been shown to be controlled at the transcriptional and post-transcriptional level. During IL-6 superinduction in breast cancer cells and HeLa cells (37) there was increased transcription in response to CHX, whereas in lung epithelial cells, fibroblasts, and Caco-2 cells (16, 39) IL-6 was increased by mRNA stabilization. Increased mRNA stability was also responsible for the superinduction of α-ENaC by CHX (17). The mechanism therefore appeared to be cell-specific. Our data showed that, in human chondrocytes, mRNA stabilization of SOX9 was increased in response to CHX and was therefore likely to be major cause of superinduction. SOX9 mRNA stability was reduced by p38 inhibition in CHX-treated cells, confirming the role p38 activation had in the mechanism. However, the reduction in mRNA stability was incomplete, suggesting that there may also be a p38-independent contribution.

This is the first demonstration of mRNA stabilization in the control of SOX9 mRNA in chondrocytes. Previous studies in chondrocytic cell lines after stimulation by IL-1β, or by small interfering RNA-mediated Bcl-2 knockdown, showed no change in SOX9 mRNA half-life (10, 49). However, evidence for SOX9 mRNA stabilization has been reported in a microarray screen examining p38 MAPK control of mRNA stability in THP-1 monocytes when treated with a p38 MAPK pathway inhibitor, SB203580 (50). Control of mRNA stability is used by

-\[\text{TABLE 1}\\
-\text{Gene sequence analysis of 3' AREs in mammalian SOX9 3' untranslated region}\]
-\text{Positions of AUUUA motifs were identified in human SOX9 mRNA (accession number NM_000346). These motifs are shown with 18 bp of both upstream and downstream sequences as described under "Experimental Procedures."}

| AUUUA motif (in bold) and surrounding sequence | Position of sequence downstream of human SOX9 mRNA stop codon in base pairs | AU content of sequence | Motif conservation |
|------------------------------------------------|--------------------------------------------------------------------------------|-----------------------|-------------------|
| UUCUCUCUCUAAAGA\text{AUUUA}AGCUAAAGGCAACUCCUGUA | 133–173 | 66 | m, r, d |
| CAUUCACAUCUAAAGGUA\text{AUUUA}ACUAAAAGUGUGAAGA | 469–509 | 76 | m, r, d |
| AAACUCUCUAAGGUA\text{AUUUA}ACUAAAAGUGUGAAGA | 628–668 | 77 | m, r, d |
| AAIUUCUCUCUAAAGA\text{AUUUA}AGCUAAAGGCAACUCCUGUA | 698–738 | 57 | m, r, d |
| AIUUCACUCUCAAGA\text{UUAAU}UUGAGAAGGUGG | 1105–1145 | 73 | m, r, d, c, h |
| UGGUACAGCAUAA\text{UUAAU}UUGAGAAGGUGG | 1265–1305 | 83 | m, r, d, c, h |
| CAGAAACUCAUAA\text{UUAAU}UUGAGAAGGUGG | 1692–1732 | 85 | m, r, d, c, h |
| UACACUCAUAA\text{CUCUG}A\text{UUAAU}UUGAGAAGGUGG | 1829–1869 | 76 | m, r, d, c, h |
overexpressing mice (55). In the conditional MKK6EE mice, p38 MAPK activity was shown to influence SOX9 transactivation activity at the protein level. Overall then, it would appear that there are multiple actions of the p38 signaling pathway on chondrocytes that can include mRNA stabilization and post-translational control of protein activity.

The present study has identified new mechanisms regulating a key chondrogenic transcription factor, SOX9, including the role of activation of the p38 MAPK pathway in determining SOX9 mRNA stability and the identification of specific 3′-ARE motifs in the SOX9 gene, which identifies a potentially important mechanism regulating SOX9 mRNA half-life in chondrogenic cells. The control of cytoskeletal organization and its influence on SOX9 expression is an important consideration in cartilage tissue engineering, and further study of mechanisms to utilize all the regulatory elements in the SOX9 gene will likely yield strategies to control the chondrocyte phenotype and drive cartilage matrix production.

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