RHO KINASE PHOSPHORYLATES SOX9 TO ENHANCE TRANSCRIPTIONAL ACTIVITY


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Purpose: Mechanical forces influence chondrocyte gene expression and biosynthetic response. The cellular response is dependent on the nature and duration of applied mechanical forces, and can range from matrix synthesis to apoptosis. Dynamic compression of moderate intensity and duration is beneficial and enhances the production of cartilage matrix components including type II collagen and aggrecan. Mechanotransduction pathways in chondrocytes that recognize applied forces and elicit appropriate biochemical outcomes are not well characterized. A current theory is that the actin cytoskeleton provides a framework for the assembly of mechanosensation mechanisms. Rho-GTPase activities regulate the actin cytoskeleton, and we have shown previously that mechanical forces activate Rho and downstream Rho Kinase (ROCK) to cause cytoskeletal rearrangements in 3D chondrocyte cultures. Sox9 is a transcription factor essential for cartilage formation and homeostasis, and it directly regulates the expression of the major collagens and proteoglycans of the cartilage extracellular matrix including type II collagen and aggrecan. We have identified a ROCK-dependent nuclear translocation of Sox9 in mechanically stimulated chondrocytes. A connection between ROCK signaling and Sox9 mRNA expression has also been found by others, but Sox9 expression and transcriptional activity are not necessarily correlated. Post-translational modifications of Sox9 may be the key to understanding its activity in cartilage. The purpose of this manuscript is to test whether a direct interaction between ROCK and Sox9 exists which affects Sox9 transcriptional activity.

Methods: SW1353 human chondrosarcoma cells were transiently transfected with various combinations of plasmids encoding for Sox9, RhoA, ROCK or kinase-dead ROCK, and a COL2A1 luciferase reporter. A ROCK consensus site within Sox9 was identified by sequence analysis. Phosphorylation was demonstrated in vitro using purified proteins, and in cyto using overexpressed proteins. Quantification was by immunoblotting with a phosphoSox9-specific antibody. Transcriptional activation of Sox9 by ROCK was measured using a Sox9-luciferase reporter assay. Inhibition of ROCK activity was with 5uM hydroxyfasudil.

Results: We identified a potential ROCK consensus site in Sox9 at serine 181.

Rho Kinase Consensus Sequence

SOX9 DKRVRKTVGNYPI S6 AKPRULSLRAST MARKS KKRFPRFPEFKFR

We found that ROCK directly phosphorylates Sox9 at this site using in vitro purified proteins. We show that overexpression of ROCK or activation of the RhoA pathway in SW1353 cells increases phosphorylation of Sox9 at this site. Serine 181 is contained within a nuclear localization signal, and its phosphorylation can enhance transcription.

Conclusions: These results demonstrate a new interaction linking Rho/ROCK activation to increased cartilage matrix production. The direct phosphorylation of the transcription factor Sox9 at one of its nuclear localization sites results in increased transcriptional activity. The organization of the chondrocyte actin cytoskeleton and chondrocyte gene expression have long appeared to be functionally linked. We believe that these results may provide a molecular mechanism to help explain this observation.

EXPRESSION PROFILING OF MOUSE FEMORAL HEAD EXPLANT CULTURES AFTER STIMULATION WITH INTERLEUKIN-1 or RETINOIC ACID

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Purpose: Cartilage explant cultures are a valuable experimental system for the analysis of the molecular mechanisms cartilage degradation. Catabolic stimulation of explants with the pro-inflammatory cytokine interleukin-1α (IL-1), or with retinoic acid (RetA), leads to degradation of aggrecan and collagen II. To further explore the mechanisms of cartilage degradation in this system, we used whole genome expression profiling to characterize RetA and IL-1 induced changes in gene expression.

Methods: Femoral head cartilage of 24-day old mice were cultured in serum-free DMEM for 2 and 4 days with either no treatment or with addition of 10uM RetA or 10ng/ml IL-1. Total RNA was extracted and cRNA generated by linear amplification (MessageAmp, Ambion), labeled with Cy3/Cy5 and hybridized to 44k whole genome microarrays (Agilent). Data was validated by quantitative PCR of selected genes.

Results: Tolidine blue staining of the explants indicated aggrecan loss in RetA treated explants by 2 days compared with control cartilage, and after 4 days when treated with IL-1. Differential gene expression of gene groups involved in cartilage catabolism, ECM formation or development was analyzed. We found that adams-4 and -5 were upregulated in both IL-1 and RetA treated cultures, while adams-9 and -15 were preferentially upregulated by RetA. A number of mmps, including mmp10, were upregulated by both agents, whereas others such as mmp3 were upregulated by IL-1 and downregulated by RetA. Timp-3 was strongly downregulated by both IL-1 and RetA, and timp-1 was upregulated by IL-1, but downregulated by RetA. Several members of the cathepsin family were also differentially regulated. Cartilage ECM molecules regulated by both IL-1 and RetA, such as collagens II, IX and XI, aggrecan, fibromodulin, link protein and matrins 1 and 3 were generally most strongly downregulated by RetA, particularly at day-4. Other SLRPs such as decorin, biglycan and prel were not significantly downregulated. In addition, differential expression of several Sox transcription factors, including Sox6, and several bmp family members was apparent.

Conclusions: The application of microarray expression profiling to mouse explant cultures stimulated with IL-1 or RetA in vitro has revealed the differential expression of many genes. Upregulation of catabolic genes such as several adams and mmp and cathepsins is new, but unsurprising information, although the differential upregulation by RetA alone, in the case of adams-9 and adams-15 is a novel finding that may provide mechanistic insights. It is important to recognize that gene expression is only part of the equation, and the application of proteomic approaches will be critical in dissecting the molecular pathways of cartilage degradation. However, our data has revealed patterns of gene expression of important candidate gene families, and of novel genes, that will be important targets of further detailed analysis.

CONTROL OF CHONDROCYTE GENE EXPRESSION BY ACTIN DYNAMICS: A NOVEL ROLE OF CHOLESTEROL/RORα SIGNALING

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Purpose: It is essential that we acquire a better understanding of chondrocyte differentiation in development as there are many parallels with osteoarthritis, a disease partially caused by the pathological differentiation of articular cartilage to hypertrophy. We have previously identified the importance of the actin cytoskeleton and regulators of actin, the family of Rho GTPases, in regulating chondrocyte maturation. More specifically, we have shown that RhoA signaling inhibits multiple stages of chondrocyte differentiation, while Rac1, Cdc42 and actin dynamics stimulate multiple aspects of chondrocyte differentiation. In order to better assess the effects of these signaling pathways, we analyzed global changes in
gene expression in response to manipulating the articular cartilage in chondrocytes.

Methods: Chondrocytes isolated from embryonic mouse growth plates were plated and grown in high density monolayer cultures and treated for 24 hours with vehicle, 10 μM Y27632 (inhibits Rho/ROCK signaling), 1 μM cytochalasin D (inhibits actin polymerization) or 50 nM jasplakinolide (promotes actin polymerization). RNA was isolated, and then hybridized to Affymetrix MOE4.0 chips. Bioinformatic analysis (MAS5.0) was performed and probe sets demonstrating a significant, at least 1.5-fold change and reliable signal were considered for further analysis. These data were compared to our previous microarray analyses of chondrocyte differentiation in vitro and in vivo (growth plate zones of microdissected tibia, time course of micromass cultures differentiating from mesenchymal cells to hypertrophy) we have previously analyzed.

Results: Inhibition of actin polymerization by cytochalasin D resulted in the most compelling data in comparison to other actin manipulations. We observed that treating growth plates with cytochalasin D resulted in very large bones consisting entirely of cells with hypertrophic morphology. Analysis of the gene set revealed that most genes that were upregulated were similarly regulated in other models of chondrocyte differentiation to hypertrophy, suggesting that cytochalasin D stimulates chondrocyte hypertrophy, a novel finding. We continued our studies by comparing gene sets with other models of chondrocyte hypertrophy to hypertrophy, suggesting that cytochalasin D stimulates chondrocyte hypertrophy, a novel finding. We continued our studies by comparing gene sets with other models of chondrocyte hypertrophy and identified the nuclear receptor, Rorα as a common gene upregulated in all 3 models studied. We found that Rorα is most highly expressed in the hypertrophic portion of the growth plate and is expressed throughout growth plates of cytochalasin D treated bones. Additionally, multiple targets of Rorα are also increased in chondrocytes treated with cytochalasin D, such as fatty acid binding protein 4, lipoprotein lipase and CD36 (a receptor previously implicated in osteoarthritis). We also show that stimulation of Rorα by its ligand cholesterol results in a phenotype similar to chondrocyte hypertrophy.

Conclusions: Identification of regulators of chondrocyte hypertrophy will be essential in preventing pathological differentiation of articular chondrocytes in osteoarthritis. Our data implicate the nuclear receptor Rorα and its activator cholesterol in stimulating chondrocyte hypertrophy. These data have far reaching implications for preventing and possibly minimizing the severity of osteoarthritis.

215 TRANSFORMING GROWTH FACTOR ALPHA AND ENDOTHELIN RECEPTOR A SIGNALING IN OSTEOARTHRITIS

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Purpose: Osteoarthritis (OA) involves the degeneration of articular cartilage by catabolic factors. Previously, we determined that transforming growth factor alpha (TGF alpha) gene expression was upregulated in articular chondrocytes in an experimental OA model. Furthermore, we demonstrated that TGF alpha suppressed chondrocyte phenotype and specifically induced expression of catabolic factors including matrix metalloproteinase-13. Thus we identified TGF alpha as a candidate causal factor in OA. Our subsequent goal was to determine downstream targets of TGF alpha. Interestingly, microarray data from our OA model indicate that endothelin receptor A (ETA) expression is also upregulated in the disease state. Since ETA has already been implicated in OA and other age-related diseases we decided to investigate whether there was a connection between TGF alpha and ETA signaling.

The purpose of the current study was to (1) examine the upregulation of ETA in our experimental OA model, (2) determine the effects of TGF alpha on ETA expression, (3) evaluate whether the catabolic effects of TGF alpha can be inhibited by blocking ETA.

Methods: RNA and histological cartilage sections from our rodent OA model were evaluated for ETA gene and protein expression by real-time PCR and immunohistochemistry respectively. Next primary articular chondrocytes were isolated from newborn Sprague Dawley rats, grown in monolayer cultures, and treated with TGF alpha (10 ng/ml) for up to 3 days. RNA was isolated from cultures and the expression of genes implicated in OA was assessed by real-time PCR. We then isolated articular cartilage organ culture explants and treated them with TGF alpha for up to 7 days. Explants were sectioned and immunostained for both ETA and inducible nitric oxide synthase (iNOS). Next we used an endothelin receptor inhibitor in an attempt to block some of TGF alpha's catabolic effects. Chondrocyte cultures were treated with either vehicle, TGF alpha, inhibitor (10 μM), or a combination of both for 48 hours. RNA was isolated and real-time PCR was performed for genes including Mmp13, Col2a1, and Agc1.

Results: Data revealed a significant increase in ETA gene expression in OA animals compared to controls. There also appeared to be deeper ETA staining in cartilage from OA animals compared to controls. In cell cultures, Cxcr4, Tgfα and Il1b gene expression did not change after treatment, while the expression of Ednra (ETA gene) and Tnfα increased in response to TGF alpha. Immunohistochemistry showed that explants treated with TGF alpha expressed more ETA than did controls. The endothelin receptor inhibitor did not appear to block TGF alpha's induction of Mmp13 or inhibition of Col2a1 and Agc1 expression by TGF alpha. Organ culture explants demonstrated an increase in iNOS expression in TGF alpha-treated tissues compared to controls. Our current cell cultures studies suggest that ETA gene expression results in increased production of nitric oxide that can be partially blocked by inhibition of endothelin signaling.

Conclusions: ETA expression was upregulated in our animal model of OA as well as in TGF alpha-treated chondrocyte cultures and articular cartilage organ cultures. Preliminary data suggest that blocking ETA may inhibit a TGF alpha-induced increase in nitric oxide production. Thus, in addition to TGF alpha, ETA may be another therapeutic target for OA treatment. While blocking ETA did not appear to alter TGF alpha's effects on Mmp13, Col2a1 and Agc1 expression, future experiments will focus on studying protein levels of these genes.

216 REGULATION OF MATRIX METALLOPROTEINASES EXPRESSION BY EPAS1 IN ARTICULAR CHONDROCYTES


Purpose: Although EPAS1 plays essential roles in hypoxia and many pathogenic conditions, the function of EPAS1 in articular chondrocytes is unclear. In this study, we characterized a role of EPAS1 in cartilage destruction.

Methods: EPAS1 expression and function in primary culture rabbit articular chondrocytes were studied by RT-PCR and qRT-PCR analysis. A role of EPAS1 in MMP expression was determined by reporter gene assay. In vivo role of EPAS1 was examined by using collagenase-induced osteoarthritis model using EPAS1 deficient (EPAS1−/−) mouse.

Results: In primary culture articular chondrocytes, EPAS1 expression was significantly increased during dedifferentiation caused by interleukin-1beta (IL-1β), epidermal growth factor, retinoic acid, or serial subculture as monolayer. Dedifferentiation of chondrocyte accompanies induction of several matrix metalloproteinases (MMPs) expression such as MMP-1, -3, -9, -12, and -13, but not MMP-2, -14, and -15. Overexpression of EPAS1 by adenoviruses-EPAS1 induced MMP-1, -3, -9, -12, and -13 expressions, whereas knockdown of EPAS1 by siRNA blocked IL-1β-induced expression of these MMPs. Regulatory mechanism of MMP expression was further examined by reporter gene containing individual MMP promoter. Reporter gene activity of MMP-1, -3, -9, -12, and -13 increased by EPAS1 transfection, and the increased activity was blocked by point mutation of EPAS1 binding site (−CGTG−) or dominant negative EPAS1. In vivo role of EPAS1 was examined by using EPAS1 deficient (EPAS1−/−) mice, and found that EPAS1 knockdown significantly reduced collagenase-induced osteoarthritis.

Conclusions: We demonstrated that EPAS1 in chondrocytes regulates expression of MMP-1, -3, -9, -12, and -13, and EPAS1 knockdown reduces collagenase-induced osteoarthritis in mice. Our results suggest that EPAS1 may play a role in cartilage destruction by modulating MMP expression.