Published in final edited form as:
J Cell Physiol. 2009 December ; 221(3): 668–676. doi:10.1002/jcp.21904.

PI3K/AKT Regulates Aggrecan Gene Expression by Modulating Sox9 Expression and Activity in Nucleus Pulposus Cells of the Intervertebral Disc

Chin-Chang Cheng[1,2], **Yoshiyasu Uchiyama**[1,3], **Akihiko Hiyama**[1,3], **Sachin Gajghate**[1], **Irving M. Shapiro**[1], and **Makarand V. Risbud**[1,*]

[1] Department of Orthopaedic Surgery, Thomas Jefferson University, 501 Curtis Building, 1015 Walnut St., Philadelphia, PA 19107, U.S.A.

[2] Department of Orthopaedic Surgery, Chang Gung Memorial Hospital at Chia-yi, Graduate Institute of Clinical Medicine Science, Chang Gung University, Taoyuan, 333, TAIWAN

[3] Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, JAPAN

Abstract

The goal of the investigation was to test the hypothesis that the PI3K/AKT signaling pathway regulates the expression of the major extracellular matrix component of the intervertebral disc, aggrecan, in nucleus pulposus cells. Primary rat nucleus pulposus cells were treated with PI3K inhibitor to measure changes in gene and protein expression. In addition, cells were transfected with various luciferase reporter plasmids to investigate mechanisms of regulation of aggrecan gene expression. We found that treatment of nucleus pulposus cells with a PI3K inhibitor, LY294002 resulted in decreased expression of aggrecan and a reduction in deposition of sulfated glycosaminoglycans. Moreover, pharmacological suppression or co-expression of dominant negative (DN)-PI3K or DN-AKT resulted in downregulation of aggrecan promoter activity. Expression of constitutively active (CA)-PI3K significantly induced aggrecan promoter activity. We observed that PI3K maintained Sox9 gene expression and activity: inhibition of PI3K/AKT resulted in decreased Sox9 expression, lowered promoter activity and mediated a reduction in Sox9 transcriptional activity. PI3K effects were independent of phosphorylation status of C-terminus transactivation domain (TAD) of Sox9. Finally, we noted that in nucleus pulposus cells, PI3K signaling controlled transactivation of p300 (p300-TAD activity), an important transcriptional coactivator of Sox9. Results of these studies demonstrate for the first time that PI3K/AKT signaling controls aggrecan gene expression, in part by modulating Sox9 expression and activity in cells of the nucleus pulposus.

Keywords

Intervertebral disc; Nucleus Pulposus; PI3K/AKT; Aggrecan; Matrix gene regulation

Introduction

The intervertebral disc permits motion between adjacent vertebrae and resists compressive loading of the spine. It consists of cells embedded in a hypoxic, gel-like nucleus pulposus,

* Correspondence to Dr. Makarand V. Risbud Department of Orthopaedic Surgery 1015 Walnut Street, Suite 501 Curtis Bldg. Thomas Jefferson University Philadelphia 19107 Tel: 215 955 1063 Fax 215 955 9159 makarand.risbud@jefferson.edu .

surrounded by a ligamentous annulus fibrosus. Cells of the nucleus pulposus are often mistakenly compared with chondrocytes although they are distinct embryologically and exist in a unique microenvironement. They secrete a complex extracellular matrix that contains high levels of the sulfated proteoglycan, aggrecan which through its interaction with cations, provides the tissue with its characteristic biomechanical and osmotic properties (Kraemer et at., 1985; Maroudas, 1970; Urban et al., 1978). In previous studies, we have shown that both hypoxia and osmotic pressure promote the expression of sulfated glycososaminoglycan (sGAG) and enhances aggrecan synthesis (Agrawal et al., 2007; Tsai et al., 2006). Accordingly, while these microenvironmental conditions favor aggrecan expression, the mechanism by which nucleus pulposus cells survive and function within the limiting and hostile confines of the intervertebral disc has not been delineated.

One clue to the mechanism of control of aggrecan synthesis is that nucleus pulposus cells exhibit a robust expression of the phosphorylated survival protein AKT (Risbud et al., 2005). Activation of phosphoinositide-3 kinase (PI3K)/AKT signaling regulates cell growth, proliferation, migration and adhesion (Cantley, 2002). In a recent study, Kita et al. (2008) have reported that in early chondrogenesis, activation of PI3K/AKT signaling promoted chondrocyte proliferation and increased sGAG deposition; inhibition of PI3K signaling resulted in decreased expression of the early chondrogenic marker genes, aggrecan, col2a1 and Sox9. Several other studies have reported a variety of functions of PI3K/AKT signaling during chondrogenesis. Hidaka et al. (2001) showed that a constitutively active form of AKT accelerated chondrogenic differentiation of ATDC5 cells. In contrast, inhibition of PI3K signaling suppressed the expression of the early chondrocytic differentiation marker *Col2a1* and the production of sulfated proteoglycans (Fujita et al., 2004). Likewise, in chondrocytic cell lines and primary articular chondrocytes, proliferation and the synthesis of sGAG is dependent on the PI3K/AKT signaling pathway (Oh and Chun, 2003; Priore et al., 2006; Qureshi et al., 2007; Starkman et al., 2005). In contrast to studies of chondrogenesis and cartilage function, the role of PI3K/AKT signaling in the regulation of aggrecan gene expression in nucleus pulposus cells has received little attention.

The goal of the investigation described herein is to test the hypothesis that the PI3K/AKT signaling pathway regulates the expression of the major extracellular component of the intervertebral disc, aggrecan. We report for the first time that PI3K/AKT signaling modulates aggrecan expression and promoter activity through Sox9, a key transcriptional regulator of chondrocyte function. This finding lends credence to the view that multiple signaling pathways contribute to regulation of aggrecan gene expression, and that hypoxia-mediated activation of PI3K signaling functionally adapts nucleus pulposus cells to their unique microenvironmental niche.

Experimental procedures

Plasmids and Reagents

Expression plasmids were provided by Dr. David Danielpour [CA-PI3K (pSG5-p110αCAAX), DN-PI3K (pSG5-p85αΔsiSH2-N) and pSG5 vector] (Song et al. 2006), Dr. Bing-Hua Jiang [DN-AKT (SR-AKT-T308A/S473A)] (Jiang et al., 1999), Dr. Peter Koopman (Sox9 reporters) (Kanai et al. 1999) and Dr. Nianli Sang [GAL4p300TAD (aa1572-2370)] (Sang et al., 2003). Construction of aggrecan reporter (−2204/+290) containing a sox core binding element (SCBE) of mouse aggrecan promoter is reported before (Tsai et al., 2006). For transactivation studies of Sox9 the binary GAL4 reporter plasmids have been described before (Tsuda et al. 2003); GAL4dbd-Sox9-TAD (aa 182-508) plasmid was provided by Dr. Hiroshi Asahara. Backbone plasmid GAL4dbd contains no TAD, but expresses the GAL4dbd. pFR-Luc (Stratagene) reporter contains the yeast GAL4-binding site, upstream of a minimal promoter and the firefly luciferase gene.

Reporter plasmid containing 89-bp *Col2a1* minimal promoter with four copies of a 48-bp *Col2a1* enhancer element (4×48-p89Luc) was provided by Dr. Benoit de Crombrugghe (Huang et al. 2000). As an internal transfection control, pRL-TK (Promega) containing *Renilla reniformis* luciferase genes was used.

Isolation and treatment of nucleus pulposus cells

Rat nucleus pulposus cells were isolated using a method reported earlier (Risbud et al., 2006). For long term culture studies, cells were cultured in alginate beads (10^6 cells/ml alginate) as well as pellets (5×10^5 cells/pellet) in DMEM with 10% FCS supplemented with ascorbate. Alginate beads and pellets were treated with 5 μM LY294002 for 4 weeks, whereas controls received carrier. For all other studies, unless otherwise mentioned, cells were maintained in monolayer culture under normoxic conditions. For short term studies, cells were treated with 30-50 μM inhibitor, this concentration was shown to specifically abolished PI3K activity without inhibiting other lipid and protein kinases (Vlahos et al., 1994).

Real Time RT-PCR analysis

Cells were cultured in 6 cm dish (4×10^5 cells/dish) one day before treatment with LY294002 (30-50 μM) for 24 h. Following treatment cells were lysed, total RNA was treated with RNase free, DNAse I and passed through RNAeasy micro columns (Quiagen). 100 ng of total RNA was used as template for real-time PCR analysis. Reactions were set up in micro capillary tubes using 1 μl RNA with 9 μl of a LightCycler FastStart DNA Master SYBR Green I mix (Roche Diagnostics, Indianapolis, IN) to which gene-specific forward and reverse PCR primers were added (Aggrecan; NM_022190, Fwd 5′-aaggactgtctatctgcacgccaa-3′, Rev 5′-tcaccacccactccgaagaagttt-3′, Sox9; XM_001081628, Fwd 5′- tgtccagccagaagcagaaatcct -3′, Rev 5′- tggtaaagttgtcgctcccactga-3′). Data was normalized using β-actin as well as 18S RNA. With each set of samples, no template control was included. PCR reactions were performed in LightCycler (Roche) according to the manufacturer's instructions. Specificity of PCR product formation was confirmed by monitoring melting peaks. Primers used were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Histological staining

Cell pellets were fixed in 4% paraformaldehyde in PBS and then embedded in paraffin. Transverse and coronal sections, 6-8 μ. in thickness, were deparaffinized in xylene, rehydrated through graded ethanol and stained with alcian blue, and with eosin and hematoxylin.

DMMB assay

The proteoglycan content of the cells cultured in alginate beads was measured as sGAG by colorimetric assay with 1-9 dimethylmethylene blue (DMMB) (Blyscan, Biolcolor Ltd., UK) with chondroitin-4-sulphate as a standard following manufacturer's instructions. Briefly, after papain digestion of cell extracts, GAGs were precipitated and stained with DMMB and staining was quantified by measuring absorbance at 656 nm. Results were calculated as GAG (μg) / total protein (μg) and expressed relative to value obtained for untreated control pellets.

Measurement of cell survival

To measure cell viability, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out as described previously (Tsai et al. 2006). Briefly, after

treatment of cell pellets with LY294002 (5 μM), MTT diluted in PBS was added to the culture medium to a final concentration of 0.5 mg/ml. At the end of the incubation period (2-4 h at 37 °C), the medium was removed, and the precipitated formazan crystals were solubilized in dimethyl sufoxide. Product formation was measured by reading the absorbance at 560 nm using a microplate reader (Tecan, Spectra Flour Plus, NC).

Western blot analysis

Cells were cultured in 10 cm dishes (7×10^5 cells/dish) one day before treatment with LY294002 (50 μM) for 24 h. Cell proteins were resolved on 8-12 % SDS-polyacrylamide gels and transferred by electroblotting to PVDF membranes (Bio-Rad, CA). The membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% tween 20) and incubated overnight at 4 °C in 3% non-fat dry milk in TBST with the anti-Sox9 (1:1000, Life Span) or anti-β-tubulin antibody (1:1000, DSHB, Iowa City, IA). Immunolabeling was detected using the enhanced chemiluminescence reagent (ECL, Amersham Biosciences).

Transfections and dual luciferase assay

Nucleus pulposus cells were transferred to 24-well plates at a density of 5×10^4 cells/well one day before transfection. For the GAL4 binary assay, cells were cotransfected with 50 ng of pFR-Luc and 50 ng of GAL4dbd or GAL4dbd-Sox9-182-508 (Sox9-TAD) or GAL4dbd-p300-1572-2370 (p300-TAD), with or without DN-PI3K/AKT or CA-PI3K/AKT1 expression plasmids or respective empty backbone vectors. For reporter assays, cells were transfected with 300 ng of Agg-Luc or Sox9-Luc or 4x48p89-Luc, with or without DN-(PI3K, AKT) or CA-PI3K and/or the backbone plasmids. For normalization, in all transfection experiments, pRL-TK plasmid containing Renilla reniformis luciferase gene was used as an internal control. LipofectAMINE 2000 (Invitrogen, CA) was used as a transfection reagent. For each transfection, plasmids were premixed with the transfection reagent. In some experiments, twenty four hours after transfection, cells were treated with LY294002 (30-50 μM). 48 hour after the initial transfection, the cells were harvested and a Dual-Luciferase™ reporter assay system (Promega) was used for sequential measurements of firefly and Renilla luciferase activities on a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA).

Statistical analysis

All measurements were performed in triplicate, data is presented as mean ± S.D. Differences between groups were analyzed by the student t test; $*p < 0.05$, $**p < 0.01$.

Results

We investigated the role of the PI3K/AKT signaling pathway in regulation of aggrecan expression and sGAG content in nucleus pulposus cells (Fig. 1). Figure 1A shows that when nucleus pulposus cells are treated with the pharmacological inhibitor of PI3K, LY294002, aggrecan mRNA expression levels are suppressed in a dose-dependent manner. Furthermore, long term treatment of cell pellets with a low dose of PI3K inhibitor, results in a decrease in sulfated proteoglycan content, as evidenced by reduced alcian blue staining (Fig. 1B). Treated cells exhibit a compacted morphology and are embedded in a disorganized extracellular matrix. We determined if there was a concurrent decrease in levels of sGAG deposition: DMMB analysis shows that treatment with LY294002 leads to a 40% decrease in sGAG deposition after 4 weeks (Fig. 1C). In addition, we determined if treatment of nucleus pulposus cells with inhibitor resulted in a decrease in cell viability. Fig. 1D shows that long term treatment with low dose of LY294002 did not decrease number of viable cells.

The functional importance of PI3K was further examined by analyzing aggrecan promoter activity using a luciferase reporter assay. Figure 2A shows reporter activity after treatment with the PI3K inhibitor LY294002 (30-50 μM). In the presence of the inhibitor, there is a substantial suppression in aggrecan promoter activity. To further explore the effect of PI3K suppression on aggrecan reporter activity, nucleus pulposus cells were transiently transfected with plasmids encoding dominant negative (DN) form of either PI3K or AKT1. Co-transfection of DN-PI3K (Fig 2B) or DN-AKT1 (Fig 2C) results in about 50% suppression of aggrecan promoter activity, compared to cells that were co-transfected with an empty backbone vector. In contrast, when cells were co-transfected with constitutively active (CA)-PI3K plasmid, there is more than a 2-fold induction in aggrecan promoter activity.

To investigate the mechanism by which PI3K signaling regulates aggrecan expression in nucleus pulposus cells, we examined Sox9 expression and function. When nucleus pulposus cells were treated with PI3K inhibitor, LY294002 there is a marked suppression in Sox9 mRNA levels (Fig. 3A). Moreover, PI3K suppression using co-transfection with DN-PI3K results in decreased activity of the Sox9 promoter (Fig. 3B). We further investigated which region of the Sox9 promoter was responsive to suppression of PI3K activity. Figure 3C and D shows that constructs harboring only the first 500 bp of the Sox9 promoter are unresponsive to LY294002 treatment. In contrast, when the reporter contained the entire 2.4 kb promoter fragment there is significant suppression. In addition, treatment with LY294002 causes a decrease in expression of Sox9 protein in nucleus pulposus cells (Fig. 3E). We then investigated the effect of PI3K signaling on transcriptional activity of Sox9 protein. In this case, we measured the activity of 4×48-p89Luc reporter that contained four copies of a Sox9-dependent 48-bp Col2a1 specific enhancer upstream of a minimal Col2a1 promoter. Figure 3F shows that treatment of nucleus pulposus cells with LY suppresses 4×48-p89Luc reporter activity. Predictably, suppression or augmentation of PI3K signaling by co-transfection with either DN-PI3K or CA-PI3K enhances or inhibits 4×48-p89 reporter activity respectively (Fig. 3G, H).

We investigated if Sox9 directly controlled aggrecan promoter activity. Figure 4B shows that co-transfection of Sox9 results in significant activation of aggrecan reporter construct that harbors a sox core-binding element (SCBE) motif. We then determined if PI3K mediated its effects on aggrecan gene expression through Sox9. Repression of aggrecan reporter activity is restored by exogenous Sox9 in presence of LY294402 (Fig. 4C). Coexpression of full length Sox9 protein results in restoration of suppression of aggrecan promoter activity mediated by DN-PI3K (Fig. 4D).

We then determined if PI3K modulated the phosphorylation status of Sox9 TAD (aa 182–508) in nucleus pulposus cells. We measured TAD activity following suppression of PI3K-AKT signaling by co-transfection with either DN-PI3K (Fig. 5A) or DN-AKT1 (Fig. 5B). We found that when PI3K-AKT signaling is blocked, Sox9-TAD activity is not affected. Likewise, when cells were co-transfected with expression plasmid encoding CA-PI3K, there is little change in the activity of Sox9-TAD in nucleus pulposus cells (not shown).

Finally, we determined if suppression of PI3K in nucleus pulposus cells resulted in decreased transactivation activity of the transcriptional co-activator p300. LY294002 suppression of PI3K activity causes a pronounced reduction in p300 TAD activity (Fig. 6A). Moreover, co-transfection with DN-PI3K also downregulates p300 TAD (aa 1572-2370) activity (Fig. 6B).

Discussion

The primary goal of this investigation was to test the hypothesis that the PI3K/AKT signaling pathway regulated aggrecan gene expression and promoted matrix synthesis activity of cells of the intervertebral disc. This study was an extension of a previous report in which we demonstrated that hypoxia increased PI3K signaling activity and elevated aggrecan gene expression in nucleus pulposus cells (Risbud et al., 2005,2006). Herein, we showed that PI3K signaling enhanced Sox9 expression and activity. In addition, this signaling pathway modulated the activity of p300, an important transcriptional coactivator of Sox9. The observation that the change in Sox9 activity was independent of its C-TAD function lent support to the view that Sox9 protein and p300 form multicomponent complexes that connect DNA-binding proteins to the transcription apparatus (Furumatsu et al., 2005). These observations provide insights into the mechanism by which PI3K/AKT signaling mediates the expression and promoter activity of aggrecan, a key protein required for nucleus pulposus function in the mechanically stressed intervertebral disc.

We evaluated the inter-relationship between the PI3K/AKT signaling pathway and aggrecan expression by gain and loss of function experiments. Suppression of PI3K activity resulted in decreased proteoglycan matrix deposition and a significant reduction in aggrecan gene expression by nucleus pulposus cells. Forced expression of DN-PI3K or DN-AKT caused a 2 fold suppression of aggrecan promoter activity. Important to note, this finding confirmed and extended studies by Ihara-Watanabe et al. (2004) who showed that PI3K was required for the differentiation of ATDC5 chondrocytes; suppression of PI3K signaling lead to delayed expression of early marker genes including aggrecan, collagen type II and the PTH/PTHrP receptor. In a similar study, it was demonstrated that PI3K was required for IGF-I dependent chondrocyte differentiation and aggrecan gene expression (Ciarmatori et al., 2007). Moreover, Ford-Hutchinson et al. (2007) reported that the conditional knockout of PTEN, a negative regulator of PI3K signaling, caused an increase in skeletal size, cortical thickness and matrix overproduction. In the studies reported herein, the aggrecan promoter activity was partially blocked following PI3K suppression. This was not surprising as there is considerable crosstalk between the PI3K/AKT pathway and other regulatory networks (Carracedo and Pandolfi, 2008). Nevertheless, the gain and loss of function studies confirmed that the PI3K/AKT signaling pathway served to regulate aggrecan expression by nucleus pulposus cells.

To further explore the mechanism by which PI3K/AKT signaling regulated aggrecan gene expression, we examined the expression of Sox9. In chondrocytes, studies by Sekiya et al. (2000) have shown that this important transcription factor drives aggrecan gene expression. In line with these findings, when PI3K/AKT function was down regulated, there was a decrease in Sox9 expression and promoter activity. Studies using successive deletion constructs suggested that the PI3K responsive element was present between −0.5 kb and −2.4 kb of the Sox9 promoter. Moreover, our results indicated that PI3K mediated regulation did not involve transcription factors such as RelA, ATF7 or GATA6 that have been implicated in Sox9 regulation in ATDC5 cells (Ushita et al., 2009). A detailed investigation to delineate molecular regulators of PI3K regulation of Sox9 promoter activity is beyond the scope of present study. Likewise, inhibition of signaling pathways lowered Sox9 protein levels and reduced Sox9 target gene expression. In a parallel study, overexpression of PI3K using CA-PI3K caused a dose-dependent increase in Sox9 target gene expression. Based on these findings, it is clear that in the nucleus pulposus, the PI3K/AKT pathway regulates Sox9 expression. With respect to aggrecan, we observed that forced expression of exogenous Sox9 when PI3K activity is blocked restored aggrecan promoter activity. Based on these findings, it is thus not unreasonable to conclude that in nucleus pulposus cells, PI3K signaling enhances aggrecan gene expression through Sox9. These

findings are in line with recent studies that show that aggrecan gene expression was increased in Sox9 transduced chondrocytes (Tew et al., 2008), although as noted by Lefebvre et al (1998) and Han and Lefebvre (2008), it is likely that upstream regulatory enhancer element and other signaling pathways may also be involved, and transduction may require Sox5, and Sox6.

To explore the mechanism by which AKT/PI3K signaling regulates aggrecan expression, we evaluated its impact on Sox9 TAD activity and p300, a transcriptional activator of Sox9. We chose to evaluate p300 as this protein coordinates and integrates the activities of Sox9 as well as other transcription factors (Tsuda et al., 2003; Furumatsu et al., 2005). In chondrocytes, Sox9 along with p300 is recruited to the col2a1 promoter and enhances transcription (Tsuda et al., 2003; Furumatsu et al., 2005). We observed that pharmacological inhibition of the PI3K/AKT signaling pathway, or co-expression of DN-PI3K caused a significant decrease in p300-TAD activity in nucleus pulposus cells. Although p300 functions as a co-activator for several other transcription factors, results of our studies and measurement of expression of several housekeeping genes (not shown) indicates that the effects on aggrecan and Sox9 promoter activity were not due to suppression of basal transcriptional activity. With respect to Sox9-TAD, previous work has shown that transactivation of Sox9 is linked to enhanced phosphorylation of residues in the TAD (aa 182-508) localized in the C terminus of the protein (Tsuda et al., 2003). Surprisingly, in nucleus pulposus cells, modulation of the PI3K/AKT signaling pathway exerted little effect on the transactivational activity of TAD-Sox9, suggesting that PI3K signaling through Sox9 did not directly influence Sox9-TAD. However, it is possible that PI3K may control Sox9 activity through phosphorylation of residues not contained within the C-TAD or through a phosphorylation independent mechanism. Based on these findings, we conclude that regulation of aggrecan gene expression by PI3K/AKT signaling in nucleus pulposus cells requires both Sox9 and p300. Future studies will be aimed at addressing the role of phosphorylation as well as determining if the regulation of aggrecan by PI3K/AKT signaling involving sox9 and p300 is specific to nucleus pulposus cells or is part of a broader mechanism.

Acknowledgments

We thank Dr. David Danielpour, Dr. Bing-Hua Jiang, Dr. Peter Koopman, Dr. Nianli Sang Dr. Hiroshi Asahara and Dr. Benoit de Crombrugghe for kindly providing necessary reagents. This work was supported by grants from the National Institutes of Health (AR050087, AR055655).

Contract grant sponsor: NIH; Contract grant number: AR050087 and AR055655

References

Agrawal A, Guttapalli A, Narayan S, Albert TJ, Shapiro IM, Risbud MV. Normoxic stabilization of HIF-1alpha drives glycolytic metabolism and regulates aggrecan gene expression in nucleus pulposus cells of the rat intervertebral disk. Am J Physiol Cell Physiol. 2007; 293:C621–C631. [PubMed: 17442734]

Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002; 296:1655–57. [PubMed: 12040186]

Carracedo A, Pandolfi PP. The PTEN-PI3K pathway: of feedbacks and cross-talks. Oncogene. 2008; 27:5527–41. [PubMed: 18794886]

Ciarmatori S, Kiepe D, Haarmann A, Huegel U, Tönshoff B. Signaling mechanisms leading to regulation of proliferation and differentiation of the mesenchymal chondrogenic cell line RCJ3.1C5.18 in response to IGF-I. J Mol Endocrinol. 2007; 38:493–08. [PubMed: 17446238]

Ford-Hutchinson AF, Ali Z, Lines SE, Hallgrímsson B, Boyd SK, Jirik FR. Inactivation of Pten in osteo-chondroprogenitor cells leads to epiphyseal growth plate abnormalities and skeletal overgrowth. J Bone Miner Res. 2007; 22:1245–59. [PubMed: 17456009]

Fujita T, Fukuyama R, Enomoto H, Komori T. Dexamethasone inhibits insulin-induced chondrogenesis of ATDC5 cells by preventing PI3K-Akt signaling and DNA binding of Runx2. J Cell Biochem. 2004; 93:374–83. [PubMed: 15368363]

Furumatsu T, Tsuda M, Yoshida K, Taniguchi N, Ito T, Hashimoto M, et al. Sox9 and p300 cooperatively regulate chromatin-mediated transcription. J Biol Chem. 2005; 280:35203–08. [PubMed: 16109717]

Han Y, Lefebvre V. L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer. Mol Cell Biol. 2008; 28:4999–13. [PubMed: 18559420]

Hidaka K, Kanematsu T, Takeuchi H, Nakata M, Kikkawa U, Hirata M. Involvement of the phosphoinositide 3-kinase/protein kinase B signaling pathway in insulin/IGF-I-induced chondrogenesis of the mouse embryonal carcinoma-derived cell line ATDC5. Int J Biochem Cell Biol. 2001; 33:1094–103. [PubMed: 11551825]

Huang W, Zhou X, Lefebvre V, de Crombrugghe B. Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer. Mol Cell Biol. 2000; 20:4149–58. [PubMed: 10805756]

Ihara-Watanabe M, Uchihashi T, Miyauchi Y, Sakai N, Yamagata M, Ozono K, et al. Involvement of phosphoinositide 3-kinase signaling pathway in chondrocytic differentiation of ATDC5 cells: application of a gene-trap mutagenesis. J Cell Biochem. 2004; 93:418–26. [PubMed: 15368367]

Jiang BH, Aoki M, Zheng JZ, Li J, Vogt PK. Myogenic signaling of phosphatidylinositol 3-kinase requires the serine-threonine kinase Akt/protein kinase B. Proc Natl Acad Sci U S A. 1999; 96:2077–81. [PubMed: 10051597]

Kanai Y, Koopman P. Structural and functional characterization of the mouse Sox9 promoter: implications for campomelic dysplasia. Hum Mol Genet. 1999; 8:691–96. [PubMed: 10072439]

Kita K, Kimura T, Nakamura N, Yoshikawa H, Nakano T. PI3K/Akt signaling as a key regulatory pathway for chondrocyte terminal differentiation. Genes Cells. 2008; 13:839–50. [PubMed: 18782222]

Kraemer J, Kolditz D, Gowin R. Water and electrolyte content of human intervertebral discs under variable load. Spine. 1985; 10:69–71. [PubMed: 3983704]

Lefebvre V, Li P, de Crombrugghe B. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are co-expressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J. 1998; 17:5718–33. [PubMed: 9755172]

Maroudas A. Distribution and diffusion of solutes in articular cartilage. Biophys. J. 1970; 10:365–79. [PubMed: 4245322]

Oh CD, Chun JS. Signaling mechanisms leading to the regulation of differentiation and apoptosis of articular chondrocytes by insulin-like growth factor-1. J Biol Chem. 2003; 278:36563–71. [PubMed: 12853454]

Priore R, Dailey L, Basilico C. Downregulation of Akt activity contributes to the growth arrest induced by FGF in chondrocytes. J Cell Physiol. 2006; 207:800–08. [PubMed: 16523491]

Qureshi HY, Ahmad R, Sylvester J, Zafarullah M. Requirement of phosphatidylinositol 3-kinase/Akt signaling pathway for regulation of tissue inhibitor of metalloproteinases-3 gene expression by TGF-β in human chondrocytes. Cell Signal. 2007; 19:1643–51. [PubMed: 17376651]

Risbud MV, Fertala J, Vresilovic EJ, Albert TJ, Shapiro IM. Nucleus pulposus cells upregulate PI3K/Akt and MEK/ERK signaling pathways under hypoxic conditions and resist apoptosis induced by serum withdrawal. Spine. 2005; 30:882–9. [PubMed: 15834331]

Risbud MV, Guttapalli A, Stokes DG, Hawkins D, Danielson KG, Schaer TP, et al. Nucleus pulposus cells express HIF-1alpha under normoxic culture conditions: a metabolic adaptation to the intervertebral disc microenvironment. J Cell Biochem. 2006; 98:152–59. [PubMed: 16408279]

Sang N, Stiehl DP, Bohensky J, Leshchinsky I, Srinivas V, Caro J. MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300. J Biol Chem. 2003; 278:14013–19. [PubMed: 12588875]

Sekiya I, Tsuji K, Koopman P, Watanabe H, Yamada Y, Shinomiya K, et al. SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line, TC6. J Biol Chem. 2000; 275:10738–44. [PubMed: 10753864]

Song K, Wang H, Krebs TL, Danielpour D. Novel roles of Akt and mTOR in suppressing TGF-beta/ALK5-mediated Smad3 activation. EMBO J. 2006; 25:58–69. [PubMed: 16362038]

Starkman BG, Cravero JD, Delcarlo M, Loeser RF. IGF-I stimulation of proteoglycan synthesis by chondrocytes requires activation of the PI 3-kinase pathway but not ERK MAPK. Biochem J. 2005; 389:723–29. [PubMed: 15801908]

Tew SR, Pothacharoen P, Katopodi T, Hardingham TE. SOX9 transduction increases chondroitin sulfate synthesis in cultured human articular chondrocytes without altering glycosyltransferase and sulfotransferase transcription. Biochem J. 2008; 414:231–36. [PubMed: 18433381]

Tsai TT, Danielson KG, Guttapalli A, Oguz E, Albert TJ, Shapiro IM, et al. TonEBP/OREBP is a regulator of nucleus pulposus cell function and survival in the intervertebral disc. J Biol Chem. 2006; 281:25416–424. [PubMed: 16772300]

Tsuda M, Takahashi S, Takahashi Y, Asahara H. Transcriptional co-activators CREB-binding protein and p300 regulate chondrocyte-specific gene expression via association with Sox9. J Biol Chem. 2003; 278:27224–29. [PubMed: 12732631]

Urban JP, Holm S, Maroudas A. Diffusion of small solutes into the intervertebral disc: as in vivo study. Biorheology. 1978; 15:203–21. [PubMed: 737323]

Ushita M, Saito T, Ikeda T, Yano F, Higashikawa A, Ogata N, et al. Transcriptional induction of SOX9 by NF-kappaB family member RelA in chondrogenic cells. Osteoarthritis Cartilage. Feb. 2009 [Epub ahead of print].

Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem. Feb.1994 269:5241–8. [PubMed: 8106507]

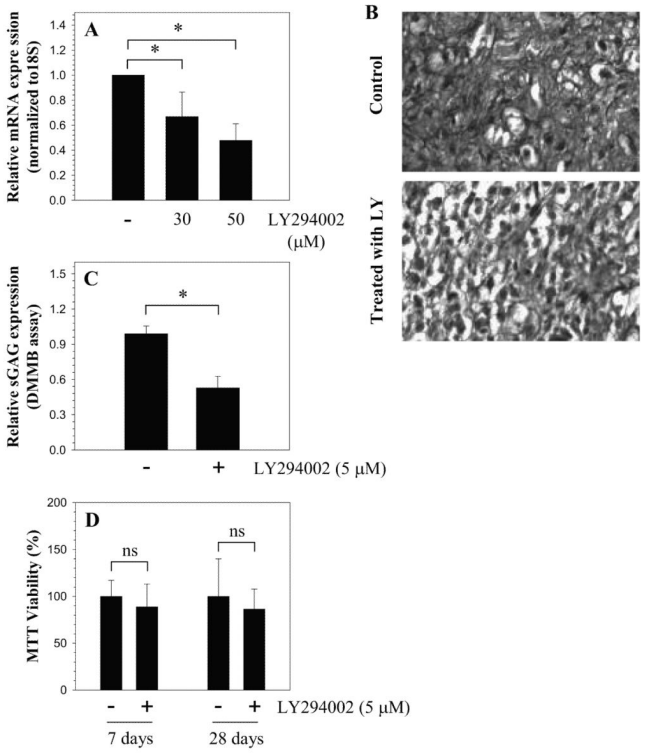

Fig. 1.

PI3K/AKT maintains aggrecan and sGAG expression in nucleus pulposus cells. A) Nucleus pulposus cells were treated with increasing concentration of LY294002 (30 and 50 μM) for 24 h and aggrecan expression analyzed by Real-time RT-PCR. PI3K inhibition caused a suppression in aggrecan mRNA levels. B) Histological analysis of nucleus pulposus cell pellets treated with or without LY294002 (5 μM) for 4 weeks. Note there was a marked decrease in alcian blue positive matrix deposition in treated cell pellets compared to controls. Mag. X 20 C) Nucleus pulposus cells cultured in alginate beads were treated with LY294002 for 4 weeks and sGAG content was measured by DMMB assay. Treatment with LY resulted in about 40% decrease in sGAG deposition by cells. D) Nucleus pulposus cell pellets treated with LY294002 for up to 4 weeks and cell viability was measured by MTT assay. No significant effect on nucleus pulposus cell viability was observed. Values shown are mean ± SD, of 3 experiments; *p<0.05.

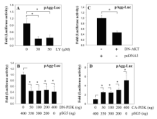

Fig. 2.

PI3K regulates aggrecan promoter activity. A) Aggrecan reporter plasmid (pAgg-Luc; -2204/+290) was transfected into rat nucleus pulposus cells along with pRL-TK vector. The cultured cells were treated with PI3K inhibitor LY294002 (30 and 50 μM) for 24 h and luciferase reporter activity was measured. Treatment with LY294002 caused a 3 fold inhibition of reporter activity. B-D) Nucleus pulposus cells were co-transfected with aggrecan reporter plasmid along with either B) DN-PI3K or C) DN-AKT or D) CA-PI3K or respective empty backbone vectors (pSG5 or pcDNA3). The cells were cultured for 48 h after transfection, and reporter activity was measured. When DN-PI3K or DN-AKT was co-transfected with the aggrecan reporter, the basal activity of the reporter was significantly suppressed. On the other hand, co-transfection with CA-PI3K resulted in increased activity of the aggrecan promoter construct. Values shown are of 3 independent experiments; mean ± SD; *$p < 0.01$.

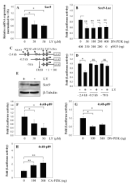

Fig. 3.

PI3K regulates Sox9 expression in nucleus pulposus cells. A) Cells were treated with increasing concentration of LY294002 (30 and 50 μM) for 24 h and Sox9 mRNA expression analyzed by Real-time RT-PCR. PI3K suppression caused a decrease in Sox9 mRNA levels. B) Cells were co-transfected with a Sox9 reporter plasmid (Sox9-Luc; −2.4/+0.3 kb) along with DN-PI3K and/or empty backbone vector pSG5. DN-PI3K significantly suppressed basal activity of the Sox9 reporter. C) Cartoon showing map of successive 5′ deletion constructs of the mouse Sox9 promoter. The transcription start site is marked as +1. Prominent transcription factor binding sites are indicated. D) Nucleus pulposus cells were transfected with each of the Sox9 promoter constructs and treated with LY294002. Inhibition of PI3K signaling resulted in selective suppression in activity of only the −2.4 kb promoter fragment. E) Western blot analysis of Sox9 expression in cells treated with LY294002 (50 μM) for 24 h. Sox9 protein levels were significantly decreased in treated cells compared to untreated controls. F-H) Nucleus pulposus cells were transfected with 4×48-p89 Col2a1 reporter plasmid and treated with LY294002 (F) or co-transfected with either DN-PI3K (G) or CA-PI3K (H) with or without backbone vector pSG5. The cells were cultured for 48 h after transfection and 24 h post inhibitor treatment and reporter activity was measured. Inhibition of PI3K signaling either by inhibitor treatment of DN-PI3K resulted in suppression of 4×48-p89 Col2a1 reporter activity. In contrast, co-transfection with CA-PI3K resulted in increased activity of this reporter. Values shown are of 3 independent experiments; mean ± SD; *$p < 0.05$, **$p < 0.01$.

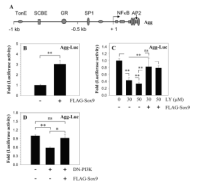

Fig. 4.

A) Cartoon depicting organization of mouse aggrecan gene promoter. The transcription start site is marked as +1. The conserved Sox core-binding element (SCBE) located at −699 bp is flanked by a TonE and GR site. B) Nucleus pulposus cells were co-transfected with aggrecan reporter and FLAG-Sox9 or empty vector. Co-expression of Sox9 results in significant induction in this reporter activity. C) Cells were co-transfected with aggrecan reporter and FLAG-Sox9 or empty vector and luciferase activity was measured following treatment with increasing concentration of LY294002 (30 μ and 50 μ. Promoter activity was significantly suppressed by LY294402 treatment. Co-expression of Sox9 resulted in restoration of promoter activity in presence of LY294002. D) Cells co-trasfected with DN-PI3K (50 ng) demonstrate suppression of aggrecan promoter activity. Addition of full length Sox9 results in restoration of aggrecan promoter activity. Values shown are of 3 independent experiments; mean ± SD; *$p < 0.05$, **$p < 0.01$.

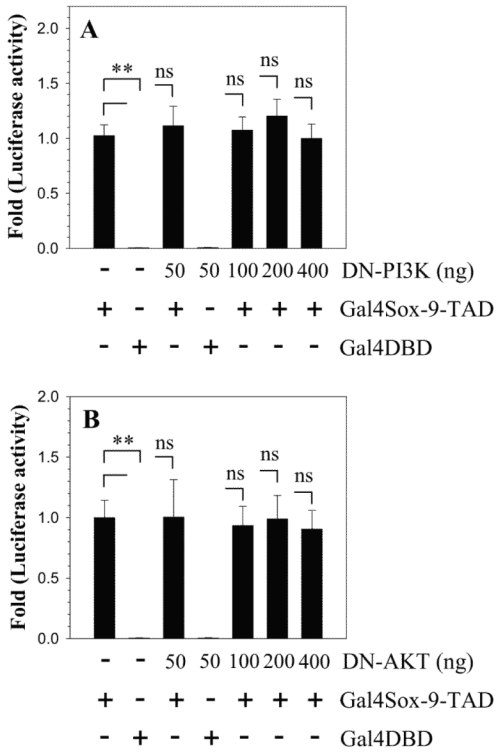

Fig. 5.

PI3K signaling does not affect Sox9-TAD activity. Nucleus pulposus cells were transfected with GAL4 binary reporter system comprising GAL4TAD-Sox9 and pFR-Luc vectors or the empty GAL4DBD vector. To examine the relationship between PI3K and TAD activity, cells were co-transfected with A) DN-PI3K and/or empty backbone vector (pSG5) or B) DN-AKT1 and/or empty vector pcDNA3 and the luciferase activity was measured 48 h after transfection. Transfection with either DN-PI3K or DN-AKT did not change Sox9-TAD activity. In all instances, when transfected with the empty GAL4DBD vector, luciferase activity was extremely low and there was no change. Data represents mean ± SD of three independent experiments; $^{**}p < 0.01$, ns = non significant.

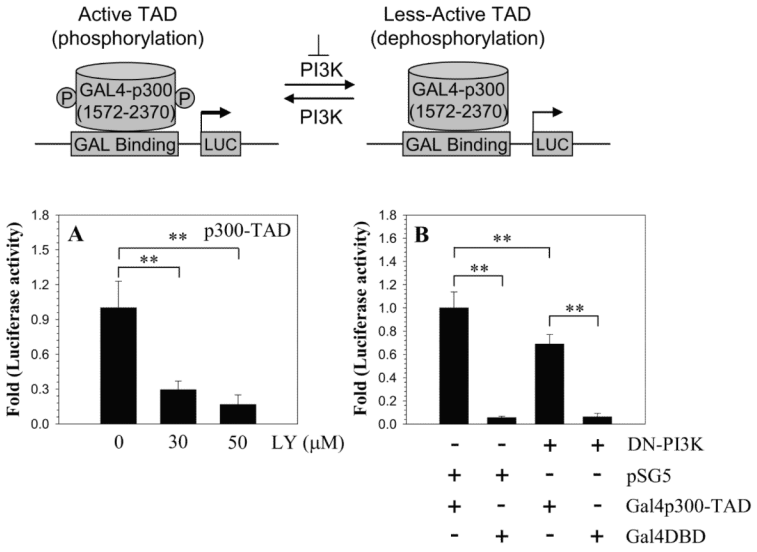

Fig. 6.

PI3K regulates transactivation activity of p300 (TAD phosphorylation) in nucleus pulposus cells. Cells were transfected with GAL4p300-TAD and pFR-Luc and A) treated with increasing concentration of LY294002 (30-50 μM) or B) co-transfected with DN-PI3K or control plasmid pSG5. Suppression of PI3K activity either by inhibitor treatment or DN-PI3K significantly decreased p300TAD activity. Background luciferase activity measured with the empty Gal4dbd was minimal. Data represents mean ± SD of three independent experiments; $^{**}p < 0.01$.