ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/40806369

Rho Kinase–Dependent Activation of SOX9 in Chondrocytes

ARTICLE *in* ARTHRITIS & RHEUMATOLOGY · JANUARY 2010

Impact Factor: 7.76 · DOI: 10.1002/art.25051 · Source: PubMed

CITATIONS
33

READS
20

5 AUTHORS, INCLUDING:

Dominik R Haudenschild
University of California, Davis
62 PUBLICATIONS **1,449** CITATIONS
SEE PROFILE

Jianfen Chen
5 PUBLICATIONS **105** CITATIONS
SEE PROFILE

Martin Lotz
The Scripps Research Institute
291 PUBLICATIONS **16,289** CITATIONS
SEE PROFILE

Available from: Martin Lotz
Retrieved on: 25 January 2016

NIH Public Access
Author Manuscript
Arthritis Rheum. Author manuscript; available in PMC 2011 January 1.

Published in final edited form as:
Arthritis Rheum. 2010 January ; 62(1): 191–200. doi:10.1002/art.25051.

Rho Kinase-Dependent Sox9 Activation in Chondrocytes

Dominik R. Haudenschild, Ph.D.[1,2], **Jianfen Chen, B.S.**[1], **Nina Pang**[2], **Martin K. Lotz, M.D.**[1], and **Darryl D. D'Lima, Ph.D., M.D.**[2]

[1] The Scripps Research Institute, Division of Arthritis Research, Scripps Clinic, La Jolla, California, 92037

[2] Shiley Center for Orthopaedic Research and Education, Scripps Clinic, La Jolla, California, 92037

Abstract

Objective—The transcription factor Sox9, directly regulates the expression of the major proteoglycans and collagens comprising the cartilage extracellular matrix. The DNA-binding activity and cellular localization of Sox9 is controlled through posttranslational modifications including phosphorylation. Rho Kinase (ROCK) has profound effects on the actin cytoskeleton, which is instrumental in determining the chondrocyte phenotype and differentiation. How ROCK is mechanistically linked to altered chondrocyte gene expression remains unknown. The purpose of this study was to test for a direct interaction between ROCK and Sox9.

Methods—Human SW1353 chondrosarcoma cells were transfected with constructs coding for RhoA, ROCK, Lim Kinase and Sox9. The interaction between ROCK and Sox9 was tested on purified proteins, and verified within a cellular context upon overexpression and activation of the Rho pathway. The effects of Sox9 transcriptional activation were quantified with a luciferase reporter plasmid containing Sox9 binding sites from the Col2a1 enhancer element.

Results—Sox9 contains a consensus phosphorylation site for ROCK. ROCK directly phosphorylates Sox9 at Serine 181 in vitro and the overexpression of ROCK or the activation of the RhoA pathway in SW1353 chondrosarcoma cells increases $Sox9^{Ser181}$ phosphorylation. ROCK causes a dose-dependent increase in the transcription of a Sox9-luciferase reporter construct, and increases phosphorylation and nuclear accumulation of Sox9 protein in response to TGF-ß and mechanical compression.

Conclusion—Taken together, these results demonstrate a new interaction that directly links ROCK to increased cartilage matrix production via activation of Sox9 in response to mechanical and growth factor stimulation.

INTRODUCTION

Cartilage is formed from condensations of mesenchymal precursor cells (1). In fetal development, the majority of the skeleton is preceded by a cartilaginous precursor template that is subsequently replaced by bone (2). In contrast, the cartilage of the joints remains unossified and provides the nearly frictionless surfaces and shock absorbing properties required for articulation. Chondrocytes of cartilaginous bone precursors and terminally differentiated chondrocytes secrete cartilage extracellular matrix, which includes type II, IX and XI collagens, aggrecan and link protein.

Address reprint requests and correspondences to: Dominik Haudenschild, 4635 Second Avenue Suite 2000, Sacramento CA 95817. Tel. 916-734-5015; Fax. 916-734-5750; email: Dominik.Haudenschild@ucdmc.ucdavis.edu.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Sox9 functions as a transcription factor essential for the formation of all cartilaginous tissues (reviewed in (3)) and it is a member of the high mobility group (HMG) superfamily of non-histone nuclear proteins (4). During embryogenesis Sox9 is a determinant of chondrocyte cell fate, and its expression precedes that of cartilage matrix proteins (5). Sox9 expression subsequently colocalizes with the expression of cartilage-specific type II collagen during development (6), and Sox9 has been shown to directly bind to the promoter and enhancer sequences of type II collagen to regulate its transcription (7–9). Sox9 also enhances the transcription of type IX (10), and XI collagens (11), aggrecan (3,12), and link protein (13), which together with hyaluronan form the major structural components of cartilage matrix. Sox9 subsequently maintains the chondrocyte phenotype by inhibiting the progression toward hypertrophy in proliferating chondrocytes (14,15).

Regulation of Sox9 activity by posttranslational modification occurs at multiple levels (16). Although ubiquitination and sumoylation sites have been identified, phosphorylation is the most widely studied posttranslational modification of Sox9. There are two consensus substrate sequences for the catalytic subunit of cyclic AMP-dependent protein kinase A (PKA-Cα) at Ser^{64} and Ser^{181}. Phosphorylation by PKA at these sites results in increased DNA-binding and transcriptional activity of Sox9 in chondrocytes (17,18). A nuclear localization signal is immediately adjacent to Serine 181 (19), and phosphorylation by PKA contributed to Sox9 nuclear localization by means of the importin-β-mediated nuclear import pathway (20). $Sox9^{Ser181}$ is also a target for phosphorylation by cyclic GMP-dependent protein kinase II (cGKII), which attenuates the ability of $Sox9^{Ser181}$ to repress hypertrophy by reducing its nuclear import (21). However, $Sox9^{Ser181}$ phosphorylation, its only known cGKII consensus site, was dispensable for both the attenuation of Sox9 activity and its reduced nuclear import, so the precise mechanism involved remains unclear (21).

Chondrocyte cell shape is linked to both phenotype and differentiation status as defined by gene expression (22–24). Cell shape is in turn dependent on the cytoskeleton and its interactions through focal adhesions with the extracellular matrix (25). Disruption of the actin cytoskeleton with cytochalasin results in a rounding of the cells and an increase in cartilage matrix production (26).

ROCK activity plays a central role in actin dynamics and has dramatic effects on cell shape (27). ROCK affects actin dynamics through the activation of Lim Kinase/Cofilin to stabilize actin filaments (28), and also through myosin light chain (MLC) and MLC phosphatase. The combined effect is enhanced actin-myosin-mediated contractility to promote morphological changes (29).

A connection between ROCK signaling, the actin cytoskeleton and chondrocyte differentiation has been found(30,31). Inhibition of ROCK signaling in monolayer cells with Y27632 caused cell rounding, increased glycosaminoglycan synthesis, and increased cortical actin organization, and these effects were linked to ROCK-dependent changes of Sox9 mRNA expression. In contrast to the results from monolayer cultures, in 3D micromass cultures ROCK inhibition increased Sox9 mRNA expression and yet reduced the expression of the Sox9 target gene aggrecan, leading to the conclusion that Sox9 mRNA expression alone is not sufficient for increased matrix gene expression (31). Similarly, inosteoarthritis the level of Sox9 mRNA expression does not necessarily correlate with the transcriptional activation of Sox9 target genes including Col2a1 (32). Consistent with this observation, Sox9 has been identified in both the cytosol and the nucleus of adult chondrocytes (32). Indeed, posttranslational modifications of Sox9 protein may be the key to understanding the regulation of its transcriptional activities in cartilage.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

To establish a direct interaction between ROCK and Sox9 proteins we demonstrate that ROCK phosphorylates $Sox9^{Ser181}$, which is adjacent to a C-terminal nuclear translocation signal within Sox9, and document a ROCK-dependent increase in Sox9 transcriptional activity using a Col2a1 luciferase reporter construct. Second, we demonstrate that this pathway is activated by two different anabolic stimuli: TGF-ß treatment, and moderate mechanical stimulation. We show a ROCK-dependent increase in the phosphorylation and nuclear accumulation of Sox9 after TGF-ß treatment. Short-term (2 hour) dynamic compression increased nuclear accumulation of Sox9 without affecting total Sox9 protein or mRNA levels. We further show that long-term (16–18 hour) dynamic compression caused an increase in total Sox9 protein, and an even greater increase in phosphorylated Sox9 protein.

Thus, ROCK activity regulates both chondrocyte cell shape and gene expression. This provides for the first time a direct link between actin and the transcription of cartilage-specific gene via the activation of Sox9 transcription factor.

MATERIALS AND METHODS

Cell Culture

SW1353 human chondrosarcoma cells (ATCC HTB-94, Manassas VA) were grown in DMEM supplemented with 10% fetal calf serum and pen/strep antibiotics. DMEM was purchased from CellGro (Mediatech Inc, Manassas VA), trypsin 10X solution was from Sigma Chemicals (Saint Louis MO), and the remaining reagents were from Invitrogen (Carlsbad, CA) unless noted otherwise. Primary human articular chondrocytes were isolated from the femoral and tibial condyles were isolated as previously described (33), and cultured in DMEM with 10% calf serum and penicillin and streptomycin antibiotics and 25ug/ml ascorbate. Cartilage for the isolation of human chondrocytes was procuredunder IRB approval by tissue banks.

Plasmid Transfections

SW1353 cells were seeded onto 6cm tissue-culture dishes at a density of 4.5×10^4 cells/cm^2 and allowed to attach overnight. Cells were then transfected with Lipofec-tamine 2000 according to the protocol provided by the manufacturer (Invitrogen). Briefly, cells were washed in Opti-MEM defined media (Invitrogen), a total of 4μg plasmid DNA was mixed with 10μl Lipofectamine reagent in 200μl of Opti-MEM, and applied to the cells. After 6 hours, the transfection media was replaced with 3ml fresh Opti-MEM and cells harvested for the respective assays after 24–48 hours. In transfection experiments with multiple plasmids, the concentration of each plasmid was held constant throughout all experimental conditions, and where necessary empty pCDNA3.1 plasmid (Invitrogen) was added to maintain a constant total plasmid concentration. The plasmid for expression of FLAG-tagged human Sox9 protein from the pCDNA5'uT-FLAG vector was a generous gift from Dr. Hiroshi Asahara at The Scripps Research Institute (TSRI). The plasmids coding for GFP:ROCK:ER and the kinase dead (KD) GFP:ROCK(KD):ER in the pBABE PURO vector were generous gifts from Dr. Michael Olson at the Beatson Institute for Cancer Research, Glasgow UK. Human LIMK and RhoA plasmids were purchased from Open BioSystems and are both in the pCMV-SPORT6 vector. The *Col2a1* luciferase reporter plasmid for Sox9 activity (12×48-pGL3P) was a generous gift from Dr. Hiroshi Asahara at TSRI.

In Vitro ROCK Assay

To determine whether ROCK and Sox9 interact *in vitro*, kinase assays were performed with purified proteins. FLAG-Sox9 plasmid was transfected into SW1353 cells as described above but in 10cm dishes, and cells were cultured 48 hours for optimal protein production.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Cells were lysed in 1ml of RIPA buffer with protease inhibitor cocktail (Sigma, St. Louis MO). FLAG-Sox9 was purified by binding 1ml of cell lysate to 5μg of anti-FLAG antibody (Sigma) immobilized onto 50μl of Protein-G agarose (Santa Cruz Biotechnology, CA) for 1 hour in RIPA buffer, and then added to the cell lysate at 4°C for 1 hour with gentile agitation. Unbound proteins were washed away with RIPA buffer. The purified FLAG-Sox9 was resuspended in kinase buffer consisting of 20mM HEPES pH 7.5, 2mM EGTA, 1mM DTT, and 5mM $MgCl_2$, with 100μM ATP added immediately before use. Recombinant human ROCK2 catalytic domain (Invitrogen) was added to a final concentration of 500ng/ml, and the kinase reaction proceeded at 30°C for 30 minutes. Adding 1x SDS-PAGE sample buffer and boiling for 5 minutes stopped the reaction.

Sox9 Phosphorylation Assay

Phosphorylated Sox9 was detected by western blotting with an antibody specific to phospho-$Sox9^{Ser181}$ (Anaspec, San Jose CA), and where applicable, the blots were stripped and re-probed with anti-total Sox9 (Chemicon/Millipore, Billerica MA) to ensure even protein loading. Primary antibodies were diluted 1:1000. Detection was with an HRP-conjugated secondary and SuperSignal West Pico ECL substrate (Pierce, Rockford IL) exposed to Kodak X-Omat film. Even protein loading was determined by quantifying protein concentration or by stripping and re-probing the blots with anti-GAPDH antibody (Ambion, Austin TX). Semi-quantitative image analysis was done on scanned digitized images using NIH-ImageJ to quantify band intensities with local background correction. Experiments were repeated at least 2–4 times and representative results shown.

Sox9 Transcriptional Activation Luciferase Assay

To determine the transcriptional activity of Sox9, a luciferase reporter plasmid was used, which contains 12 repeats of a 48bp *Col2a1* intron 1 enhancer element and is known to be activated by Sox9 (34). Constant amounts of luciferase reporter (150ng), renilla control (15ng) and Sox9-FLAG (150pg) plasmids were co-transfected with various amounts of ROCK:ER or ROCK(KD):ER plasmids (ranging from 0 to 170ng). The total amount of transfected DNA was held constant at 336ng by adding empty pCDNA3.1 where necessary. The transfected SW1353 cells were cultured for 48 hours in the presence of 1μM Tamoxifen to activate the ROCK:ER and ROCK(KD):ER proteins. Cells were lysed and luciferase and renilla activities were analyzed using the Dual-Luciferase reporter assay system (Promega, Madison WI) according to protocol. The assays were performed in triplicate in 48-well cell culture dishes.

Processing of 3D Chondrocyte Cultures

Primary human articular chondrocytes obtained from the femoral and tibial condyles were isolated as previously described (33). The final cell density was 5×10^6 cells/ml in hydrogel discs 6mm in diameter and 2.3 mm high. Cells were cultured with one gel per well in 24-well plates, in DMEM supplemented with 10% fetal calf serum (FCS) and 25μM ascorbic acid. Media was changed every 2 to 3 days. For western blotting analysis, chondrocytes embedded in 3% agarose hydrogel (Fluka, Switzerland) were directly added to volume 4x SDS-PAGE sample buffer, then boiled 5 minutes and loaded onto SDS-PAGE gels before the agarose re-solidified. For Sox9 nuclear localization, chondrocytes embedded in 2% alginate hydrogel (NovaMatrix, Norway) were fixed in 3.7% neutral buffered formaldehyde (Fisher Scientific) for 45 minutes at room temperature. After fixation, the alginate was dissolved in 100mM EDTA for 5 minutes and the chondrocytes gently centrifuged onto glass slides with a Cytospin Cytocentrifuge (Thermo Scientific) for subsequent immunofluorescence processing. Blocking was with PBS-3% BSA, and anti-Sox9 was applied at 3ug/ml overnight, and visualized with an Alexa-488 conjugated secondary (Invitrogen). ToPro-3 was used as a nuclear counters-tain.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Dynamic compression

To apply physiological loading to the cells, the hydrogel-embedded chondrocytes were placed in a custom designed polysulfone chamber and subjected to continuous sinusoidal cyclic compression of 5% amplitude above and below a 10% offset (5%–15% strain) at a frequency of 0.5Hz, as described previously (35).

Quantification of Nuclear Sox9

Images of chondrocytes were obtained on a Zeiss LSM-510 confocal microscope with a 63× water-immersion objective, using the motorized stage and tiling function to image a large region of the slide. A combination of ImageJ and CellProfiler (36) image analysis routines were used to identify individual nuclei from the ToPro3 nuclear stain, then quantify the intensity of nuclear Sox9 for each cell. JMP 7.0 (SAS Institute, Cary, NC) was used for statistical analysis using ANOVA with Tukey's Post-hoc corrections for multiple comparisons.

RESULTS

ROCK consensus sequence in Sox9

The substrate sequence for ROCK has recently been thoroughly characterized, and from 18 different substrates a consensus sequence of RXXS/T or RXS/T has been defined (37). $Sox9^{Ser181}$ is within a ROCK consensus sequence (Figure 1A), and might therefore serve as a substrate for phosphorylation by ROCK.

ROCK Phosphorylation of $Sox9^{Ser181}$ in vitro

To test whether the potential ROCK recognition sequence we identified can actually serve as a substrate for ROCK, an *in vitro* kinase assay was performed with purified proteins. Human Sox9 was overexpressed as a FLAG epitope-tagged fusion protein in SW1353 chondrosarcoma cells and was purified by immunoprecipitation with an anti-FLAG antibody. Recombinant human ROCK active domain was then added and the kinase reaction was allowed to proceed for 30 minutes. A small amount of basal Sox9 phosphorylation was observed, and this was greatly increased in the presence of exogenously added Sox9 protein (Figure 1B). These results demonstrate that a direct interaction occurs between ROCK and Sox9, and that $Sox9^{Ser181}$ is a target substrate for phosphorylation by ROCK.

ROCK Phosphorylation of Sox9 in intact cells

To test for an interaction between ROCK and Sox9 in a cellular context, we transfected SW1353 chondrosarcoma cells with a conditionally active ROCK:ER (Estrogen Receptor) construct that is constitutively expressed in an inactive form, and that becomes activated in the presence of the estrogen analog Tamoxifen (38). As a control, an identical construct was transfected, which contains a single point mutation in the ROCK active domain rendering it kinase-dead (KD) even in the presence of Tamoxifen (ROCK(KD):ER) (38). FLAG-Sox9 was co-transfected, and the amount of phosphorylated Sox9 was measured in whole cell lysate by western blotting with an anti-phospho-$Sox9^{Ser181}$ antibody. Expression and activation of the ROCK construct increased the phosphorylation levels of $Sox9^{Ser181}$ by >50% within 2 hours (Figure 2A). Since the transfection efficiency was rather low, it is likely that the observed 50% change under-estimates the actual increase in phospho-Sox9 in individual transfected cells. No increase in phosphorylated Sox9 was seen with the kinase-dead mutant construct. This data demonstrates that $Sox9^{Ser181}$ is a target for phosphorylation by ROCK in an intracellular environment.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Sox9^{Ser181} phosphorylation is a downstream of RhoA pathway activation and independent of LIM Kinase

ROCK is a major effector protein downstream of Rho activation and provides a link to actin remodeling via LIM Kinase and Cofilin (39,40). We next determined whether activation of the RhoA pathway influences Sox9 phosphorylation. To test this hypothesis, plasmids coding for FLAG-Sox9 and RhoA were co-transfected. As negative controls, Sox9 was co-transfected with an empty vector (pCDNA3.1). Sox9 phosphorylation increased approximately 75% in the presence of overexpressed RhoA (Figure 2B). ROCK signals through LIM kinase activation to inhibit cofilin activity and therefore alter actin filament dynamics. To exclude the possibility that LIM kinase or its downstream pathways mediate Sox9 phosphorylation upon Rho/ROCK activation, we co-transfected a plasmid for LIM Kinase together with Sox9. No increase in Sox9 phosphorylation was observed when LIM Kinase was co-transfected either alone or together with Sox9 plasmid. These results provide evidence that activation of the RhoA pathway increases Sox9^{Ser181} phosphorylation in a cellular context through a new pathway not mediated by LIM kinase. These observations lend credence to hypothesis that activation of the ROCK pathway leads to a direct phosphorylation of Sox9^{Ser181}.

Dose-dependent induction of Sox9 transcriptional activity by ROCK

The previous data indicated that ROCK directly interacts with Sox9 to phosphorylate Serine 181. To determine the effect of ROCK on the transcriptional activity of Sox9 on chondrocyte extracellular matrix gene expression, we used a luciferase reporter construct that contains 12 repeats of a 48bp Sox9-binding sequence from the human type 2 collagen intron 1 enhancer (34). Co-transfections of this reporter with a constant amount of Sox9 and increasing amounts of ROCK showed a dose-dependent increase in Sox9 transcriptional activity with increasing ROCK (Figure 3). As negative controls, similar amounts of the kinase-dead ROCK(KD):ER mutant or pCDNA3.1 control plasmid had no effect on Sox9 transcriptional activity. These results support the hypothesis that ROCK activity enhances Sox9-mediated transcription.

ROCK-dependent Sox9 phosphorylation with growth factor treatment

To test for interaction between ROCK and Sox9 in a biological context, we treated SW1353 chondrosarcoma cells with 10ng/ml TGF-β_1 for 20 hours. After TGF-ß$_1$ treatment, we were able to detect endogenous phosphorylated Sox9 by immunoblotting. However, when we inhibited ROCK activity by adding 5uM hydroxyfasudil for 2 hours prior to and during the TGF-ß$_1$ treatment, we consistently saw reduced and sometimes undetectable levels of phosphorylated Sox9 (Figure 4A).

ROCK-dependent Sox9 nuclear localization with growth factor treatment

The ROCK consensus sequence on Sox9 is adjacent to a nuclear localization signal that has been described previously (17). Having shown that there is a ROCK-dependent Sox9 phosphorylation in TGF-ß$_1$-treated chondrocytes, we wanted to test whether this caused increased nuclear Sox9 localization. Sox9 was detected by immunofluorescence and nuclear localization was determined by colocalization with the ToPro3 nuclear stain. We detected increased nuclear Sox9 within 30 minutes of TGF-ß$_1$ treatment, which reached a maximum after two to four hours and remained elevated for at least 24 hours with continuous TGF-ß$_1$ exposure. The increase in nuclear Sox9 was not seen when TGF-ß$_1$ was added in the presence of ROCK inhibitor (Figure 4B). ROCK inhibitor alone did not have any effect on nuclear Sox9. We did not detect changes in either Sox9 mRNA or total protein levels (data not shown). These results demonstrate that ROCK-dependent phosphorylation of

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

endogenous Sox9 occurs in a biologically relevant context and results in increased nuclear localization of the transcription factorin human chondrocytes.

ROCK-dependent Sox9 nuclear localization upon short-term dynamic compression

Moderate physical stimulation is an anabolic signal for 3D cultured chondrocytes which results in enhanced expression of Sox9 target genes including cartilage matrix collagens and proteoglycans. We wanted to determine whether the newly identified ROCK-Sox9 interaction is activated by dynamic compression of alginate-embedded chondrocytes. We observed increased nuclear Sox9 after 2 hours of dynamic compression, and this increase was inhibited by performing the dynamic compression in the presence of ROCK inhibitor (Figure 5A). ROCK inhibitor alone did not have any effect on nuclear Sox9, and we did not detect changes in the total amount of Sox9 mRNA or protein at this time point. Long-term dynamic compression of 16–18 hours resulted in increased levels of Sox9 protein and an even larger increase in the amount of phosphorylated Sox9 protein (Figure 5B).

DISCUSSION

Chondrocyte phenotype and gene expression are greatly affected by cell shape as determined by the actin cytoskeleton (26,41). Several lines of research have identified Sox9 as a master regulator of chondrocyte gene expression (42), and ROCK as a major pathway regulating cell shape by means of the actin cytoskeleton (43,44). To elucidate the mechanisms linking these two observations, this study addressed the hypothesis that Sox9 is a direct target of phosphorylation by ROCK. We identified a potential ROCK consensus sequence at $Sox9^{Ser181}$, and demonstrated that ROCK phosphorylates Sox9 at this site *in vitro*. We showed that this interaction occurs in a cellular context and upon activation of the RhoA pathway. We further showed a ROCK-dependent increase in Sox9 transcriptional activity using a luciferase reporter construct containing Sox9 binding sites from *Col2a1*. Finally, we provide evidence that this that this pathway is activated in two different anabolic stimuli relevant to cartilage, namely dynamic compressive forces and TGF-ß treatment.

A possible mechanism through which $Sox9^{Ser181}$ phosphorylation enhances Sox9 transcriptional activation is by imparting a negative charge to the nuclear localization signal, which then facilitates binding to nucleocytoplasmic transport factor importin-β (45). This increases Sox9 nuclear import and thus has the potential of increasing Sox9 transcriptional activity without affecting Sox9 abundance at the protein or mRNA levels. Post-translational regulation of Sox9 activity is supported by the seemingly contradictory observation that Col2a1 transcription is not necessarily correlated to the level of Sox9 mRNA expression in adult cartilage and osteoarthritis (32). Our demonstration of a direct ROCK-Sox9 interaction is further supported by previous work in which $Sox9^{Ser181}$ phosphorylation and transcriptional activity was suppressed by the ROCK inhibitor Y27632 (31), although in that study they did not provide a mechanism for this observation. A post-translational regulatory mechanism has been described for Sox9 activity in Sertoli cell differentiation via $Sox9^{Ser181}$ phosphorylation by cAMP-PKA (20). cAMP-PKA is structurally related to ROCK kinase, and it cannot be ruled out that the low levels of basal Sox9 activity observed even in the presence of ROCK inhibitors (in Figures 3 and 4B) are due to phosphorylation of $Sox9^{Ser181}$ by cAMP-PKA. However, our results demonstrate that a direct interaction between ROCK and Sox9 which occur upon ROCK activation.

The implications of ROCK-dependent Sox9 phosphorylation are likely to be context specific, and may in fact lead to different outcomes in mature chondrocytes versus in chondroprogenitors or chondrosarcoma cell lines such as SW1353. The cellular models employed may also affect the outcomes, depending on whether the cells are in a proliferative mode in monolayer cultures or undergoing differentiation in 3D or micromass cultures. Sox9

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

acts a part of a larger transcription factor complex containing other proteins that are also regulated in response to the activation of additional signaling pathways. It is the state of the larger transcriptional complex that ultimately determines its specificity and transcriptional activity in a context specific manner. For example, in epithelial cells undergoing mesenchymal transition, TGF-ß can rapidly activate RhoA and ROCK signaling and these events are required for the effects of TGF-ß (46). TGF-ß treatment of chondrocytes increases Sox9 activity via Smad3-dependent recruitment of CBP/p300 and Sox9 into a transcriptional complex on cartilage specific genes (47). However, the linker region of Smad3 itself is a target for phosphorylation by ROCK in epithelial cells, and its phosphorylation state affects Smad-dependent transcription (48). Sox9 also directly interacts with the transcriptional co-activator PGC-1α to promote chondrogenesis (49). In chondrocytes and mesenchymal chondroprogenitors the implications of ROCK-dependent Sox9 phosphorylation on the assembly and activity of the larger multi-protein transcriptional complex remain unknown and warrant further investigation.

In this study we show that ROCK pathway activation leads to Sox9 phosphorylation, nuclear accumulation, and transcriptional activation. One potential upstream event leading to ROCK activation is TGF-ß, a major anabolic pathway in cartilage that also affects actin cytoskeletal dynamics (50). In Figure 4 we show that TGF-ß treatment causes a ROCK-dependent increase in endogenous levels of phosphorylated Sox9, and that in 3D cultures of low-passage human chondrocytes TGF-ß treatment caused increased nuclear Sox9 accumulation. Consistent with this, the anabolic effect of TGF-ß on proteoglycan synthesis was partially blocked by ROCK inhibitor Y27632 (data not shown). We have previously identified mechanical stimulation as another anabolic upstream event leading to Rho activation (33,35). In Figure 5, we show that dynamic mechanical compression caused a ROCK-dependent increase in nuclear Sox9 accumulation within two hours. In Figure 6 we schematically present an updated model in which activation of Rho and ROCK from mechanical or other stimuli leads to thedirect phosphorylation and nuclear accumulation of Sox9 by ROCK to enhance matrix gene transcription. This occurs in addition to the previously established pathways affecting actin remodeling via LIM Kinase and cofilin (27).

In summary, ROCK activity plays a central role in determining actin organization (27), and the organization of actin has been linked to chondrocyte gene expression (22–24) via unknown mechanisms. Our demonstration that ROCK activity modulates chondrocyte gene expression via direct Sox9 phosphorylation and activation establishes a mechanistic foundation for these observations and opens additional therapeutic options in the treatment of arthritis.

Acknowledgments

Grant Supporters:

This work was funded through NIH grants AG07996 and AG033409 (MKL) and a generous donation from Donald P. and Darlene V. Shiley. Plasmids for ROCK were a generous gift from Dr. Michael Olson, and FLAG-Sox9 and the 12×48-pGL3P COL2A1 luciferase reporters were generous contributions from Dr. Hiroshi Asahara.

References

1. Goldring MB, Tsuchimochi K, Ijiri K. The control of chondrogenesis. J Cell Biochem. 2006; 97(1): 33–44. [PubMed: 16215986]
2. Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003; 423(6937):332–6. [PubMed: 12748651]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

3. Lefebvre V, Dumitriu B, Penzo-Mendez A, Han Y, Pallavi B. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. Int J Biochem Cell Biol. 2007; 39(12):2195–214. [PubMed: 17625949]
4. Goodwin GH, Sanders C, Johns EW. A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. Eur J Biochem. 1973; 38(1):14–9. [PubMed: 4774120]
5. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. Sox9 is required for cartilage formation. Nat Genet. 1999; 22(1):85–9. [PubMed: 10319868]
6. Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, et al. The Sry-related gene Sox9 is expressed duringchondrogenesis in mouse embryos. Nat Genet. 1995; 9(1):15–20. [PubMed: 7704017]
7. Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, et al. SOX9 directly regulates the type-II collagen gene. Nat Genet. 1997; 16(2):174–8. [PubMed: 9171829]
8. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Mol Cell Biol. 1997; 17(4):2336–46. [PubMed: 9121483]
9. Ng LJ, Wheatley S, Muscat GE, Conway-Campbell J, Bowles J, Wright E, et al. SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. Dev Biol. 1997; 183(1):108–21. [PubMed: 9119111]
10. Zhang P, Jimenez SA, Stokes DG. Regulation of human COL9A1 gene expression. Activation of the proximal promoter region by SOX9. J Biol Chem. 2003; 278(1):117–23. [PubMed: 12399468]
11. Bridgewater LC, Lefebvre V, de Crombrugghe B. Chondrocyte-specific enhancer elements in the Col11a2 gene resemble the Col2a1 tissue-specific enhancer. J Biol Chem. 1998; 273(24):14998–5006. [PubMed: 9614107]
12. 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(15):10738–44. [PubMed: 10753864]
13. Kou I, Ikegawa S. SOX9-dependent and -independent transcriptional regulation of human cartilage link protein. J Biol Chem. 2004; 279(49):50942–8. [PubMed: 15456769]
14. Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. 2002; 16(21):2813–28. [PubMed: 12414734]
15. Okubo Y, Reddi AH. Thyroxine downregulates Sox9 and promotes chondrocyte hypertrophy. Biochem Biophys Res Commun. 2003; 306(1):186–90. [PubMed: 12788086]
16. Kawakami Y, Rodriguez-Leon J, Belmonte JC. The role of TGFbetas and Sox9 during limb chondrogenesis. Curr Opin Cell Biol. 2006; 18(6):723–9. [PubMed: 17049221]
17. 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(11):4149–58. [PubMed: 10805756]
18. Huang W, Chung UI, Kronenberg HM, de Crombrugghe B. The chondrogenic transcription factor Sox9 is a target of signaling by the parathyroid hormone-related peptide in the growth plate of endochondral bones. Proc Natl Acad Sci U S A. 2001; 98(1):160–5. [PubMed: 11120880]
19. Preiss S, Argentaro A, Clayton A, John A, Jans DA, Ogata T, et al. Compound effects of point mutations causing campomelic dysplasia/autosomal sex reversal upon SOX9 structure, nuclear transport, DNA binding, and transcriptional activation. J Biol Chem. 2001; 276(30):27864–72. [PubMed: 11323423]
20. Malki S, Nef S, Notarnicola C, Thevenet L, Gasca S, Mejean C, et al. Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation. Embo J. 2005; 24(10):1798–809. [PubMed: 15889150]
21. Chikuda H, Kugimiya F, Hoshi K, Ikeda T, Ogasawara T, Shimoaka T, et al. Cyclic GMP-dependent protein kinase II is a molecular switch from proliferation to hypertrophic differentiation of chondrocytes. Genes Dev. 2004; 18(19):2418–29. [PubMed: 15466490]
22. Benya PD, Padilla SR, Nimni ME. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell. 1978; 15(4):1313–21. [PubMed: 729001]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

23. Dessau W, Vertel BM, von der Mark H, von der Mark K. Extracellular matrix formation by chondrocytes in monolayer culture. J Cell Biol. 1981; 90(1):78–83. [PubMed: 6788783]
24. Glowacki J, Trepman E, Folkman J. Cell shape and phenotypic expression in chondrocytes. Proc Soc Exp Biol Med. 1983; 172(1):93–8. [PubMed: 6828458]
25. Woods A, Wang G, Beier F. Regulation of chondrocyte differentiation by the actin cytoskeleton and adhesive interactions. J Cell Physiol. 2007; 213(1):1–8. [PubMed: 17492773]
26. Zanetti NC, Solursh M. Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton. J Cell Biol. 1984; 99(1 Pt 1):115–23. [PubMed: 6539780]
27. Ridley AJ. Rho family proteins: coordinating cell responses. Trends Cell Biol. 2001; 11(12):471–7. [PubMed: 11719051]
28. Sorokina EM, Chernoff J. Rho-GTPases: new members, new pathways. J Cell Biochem. 2005; 94(2):225–31. [PubMed: 15543593]
29. Olson MF. Applications for ROCK kinase inhibition. Curr Opin Cell Biol. 2008
30. Woods A, Wang G, Beier F. RhoA/ROCK signaling regulates Sox9 expression and actin organization during chondrogenesis. J Biol Chem. 2005; 280(12):11626–34. [PubMed: 15665004]
31. Woods A, Beier F. RhoA/ROCK signaling regulates chondrogenesis in a context-dependent manner. J Biol Chem. 2006; 281(19):13134–40. [PubMed: 16565087]
32. Aigner T, Gebhard PM, Schmid E, Bau B, Harley V, Poschl E. SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes. Matrix Biol. 2003; 22(4):363–72. [PubMed: 12935820]
33. Haudenschild DR, Nguyen B, Chen J, D'Lima DD, Lotz MK. Rho kinase-dependent CCL20 induced by dynamic compression of human chondrocytes. Arthritis Rheum. 2008; 58(9):2735–42. [PubMed: 18759278]
34. 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(42):35203–8. [PubMed: 16109717]
35. Haudenschild DR, D'Lima DD, Lotz MK. Dynamic compression of chondrocytes induces a Rho kinase-dependent reorganization of the actin cytoskeleton. Biorheology. 2008; 45(3–4):219–28. [PubMed: 18836226]
36. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol. 2006; 7(10):R100. [PubMed: 17076895]
37. Kang JH, Jiang Y, Toita R, Oishi J, Kawamura K, Han A, et al. Phosphorylation of Rho-associated kinase (Rho-kinase/ROCK/ROK) substrates by protein kinases A and C. Biochimie. 2007; 89(1): 39–47. [PubMed: 16996192]
38. Croft DR, Olson MF. Conditional regulation of a ROCK-estrogen receptor fusion protein. Methods Enzymol. 2006; 406:541–53. [PubMed: 16472686]
39. Schwartz M. Rho signalling at a glance. J Cell Sci. 2004; 117(Pt 23):5457–8. [PubMed: 15509861]
40. Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol. 2003; 4(6):446–56. [PubMed: 12778124]
41. Daniels K, Solursh M. Modulation of chondrogenesis by the cytoskeleton and extracellular matrix. J Cell Sci. 1991; 100 (Pt 2):249–54. [PubMed: 1757484]
42. de Crombrugghe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W. Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol. 2000; 19(5):389–94. [PubMed: 10980415]
43. Hall A. Rho GTPases and the actin cytoskeleton. Science. 1998; 279(5350):509–14. [PubMed: 9438836]
44. Hall A. Rho GTPases and the control of cell behaviour. Biochem Soc Trans. 2005; 33(Pt 5):891–5. [PubMed: 16246005]
45. Moroianu J. Nuclear import and export: transport factors, mechanisms and regulation. Crit Rev Eukaryot Gene Expr. 1999; 9(2):89–106. [PubMed: 10445152]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

46. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, et al. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell. 2001; 12(1):27–36. [PubMed: 11160820]

47. Furumatsu T, Tsuda M, Taniguchi N, Tajima Y, Asahara H. Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. J Biol Chem. 2005; 280(9):8343–50. [PubMed: 15623506]

48. Kamaraju AK, Roberts AB. Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor-beta-mediated Smad-dependent growth inhibition of human breast carcinoma cells in vivo. J Biol Chem. 2005; 280(2):1024–36. [PubMed: 15520018]

49. Kawakami Y, Tsuda M, Takahashi S, Taniguchi N, Esteban CR, Zemmyo M, et al. Transcriptional coactivator PGC-1alpha regulates chondrogenesis via association with Sox9. Proc Natl Acad Sci U S A. 2005; 102(7):2414–9. [PubMed: 15699338]

50. Bhowmick NA, Ghiassi M, Aakre M, Brown K, Singh V, Moses HL. TGF-beta-induced RhoA and p160ROCK activation is involved in the inhibition of Cdc25A with resultant cell-cycle arrest. Proc Natl Acad Sci U S A. 2003; 100(26):15548–53. [PubMed: 14657354]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

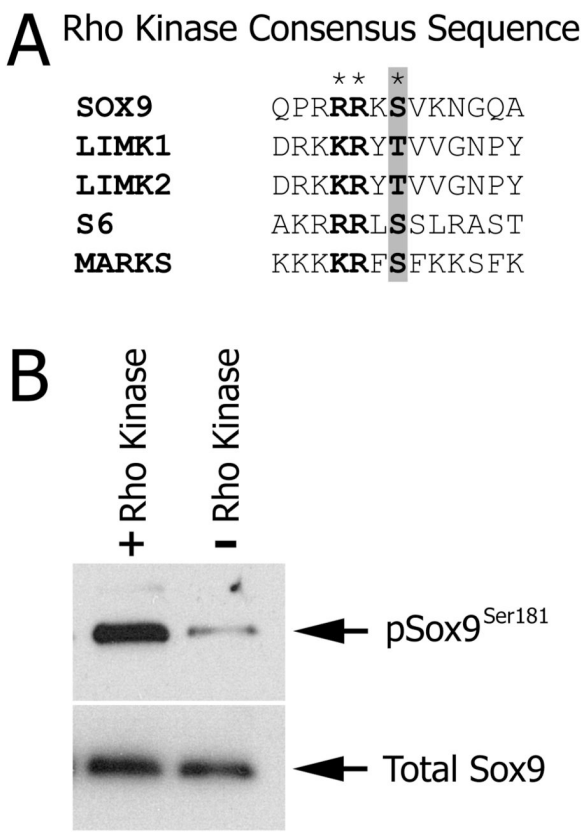

Figure 1.

A, Sox9 contains a ROCK consensus sequence. Amino-acid alignments of Sox9 with several known ROCK substrates. The consensus serine and threonine phosphorylation residues are highlighted in grey, and the commonly required basic residues at positions -2 and -3 are boldface. **B,** ROCK directly interacts with and phosphorylates Sox9^{Ser181} *in vitro*. FLAG-Sox9 expressed in SW1353 chondrosarcoma cells and purified by immunoprecipitation with anti-FLAG serves as a substrate for phosphorylation by recombinant human ROCK *in vitro.* Phosphoryla-tion was measured by western blotting with an anti-phospho-Sox9^{Ser181} antibody, and the blot was stripped and re-probed with anti-total Sox9 to show even protein loading.

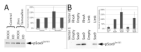

Figure 2.

A, ROCK phosphorylates Sox9 *in intact cells*. Inducible ROCK:ER and ROCK(KD):ER were cotransfected with Sox9 into SW1353 chondrosarcoma cells, and ROCK was activated for 2 hours by the addition of Tamoxifen. The amount of phosphorylated Sox9 present in the cell lysate was assayed by western blotting with an anti-phospho-Sox9^{Ser181} antibody. Total protein in each lane was constant. Results shown are typical of 3 independent experiments. The inset graphically shows image densitometry, error bars represent standard deviations. **B,** Sox9 phosphorylation is downstream of RhoA activation and independent of Lim kinase. RhoA was co-transfected with Sox9 and the amount of phosphorylated Sox9^{Ser181} was assayed by western blotting of total cell lysate 24 hours after transfection. Sox9^{Ser181} phosphorylation was significantly increased only when RhoA was co-transfected ($p<0.05$). As negative controls, Sox9 plasmid was co-transfected with empty pCDNA3.1 vector (EV). Co-transfection with LIM kinase plasmid did not affect Sox9 phosphorylation ($p=.618$). Results shown are typical of 4 independent experiments. The inset graphically shows image densitometry, error bars represent stan-dard deviations.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

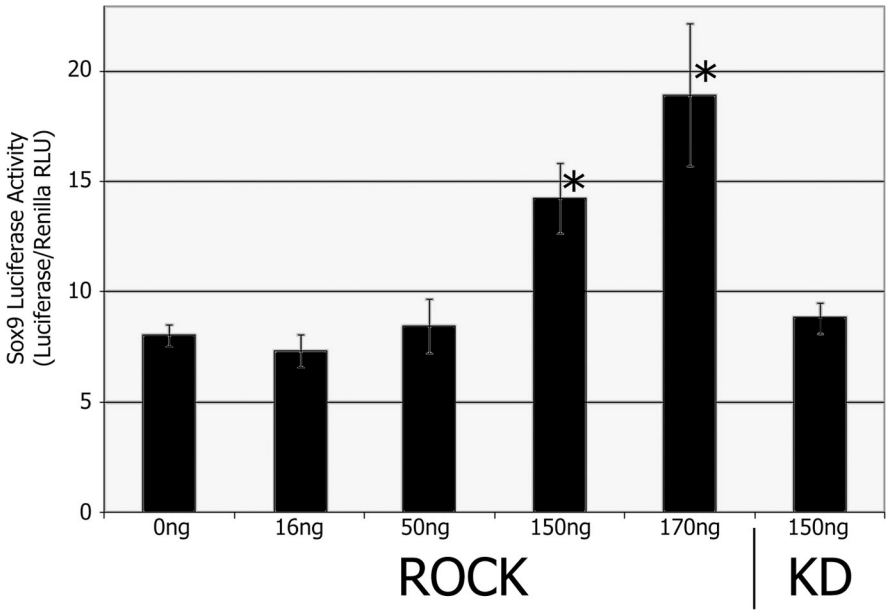

Figure 3. ROCK causes a dose-dependent increase in Sox9 transcriptional activity

Co-transfection of various amounts of ROCK:ER or a kinase-dead mutant ROCK(KD):ER with a *col2a1*-Luciferase reporter containing Sox9 binding sites into SW1353 chondrosarcoma cells in monolayer culture. After 48 hours cells were assayed for luciferase activity. The total amount of plasmid was held constant by the addition of pCDNA3.1 empty vector, and transfection efficiency was controlled for by assaying renilla activity in each sample. The amount of ROCK:ER or ROCK(KD):ER plasmid transfected is indicated in the X-axis legend. Error bars represent standard deviation of 3 replicate transfections within a typical experiment, * indicates $p<0.05$ compared to control.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

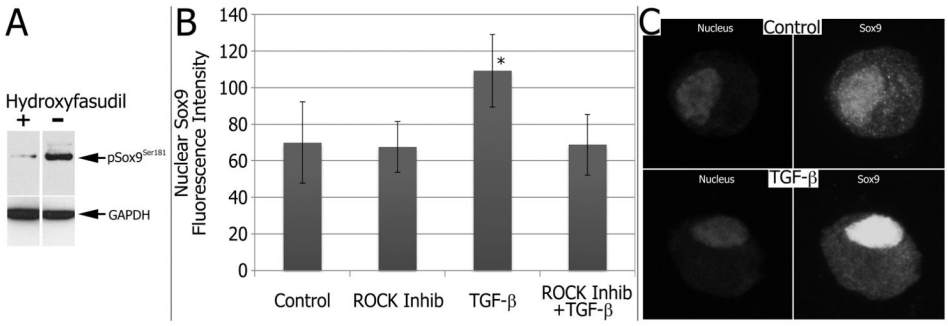

Figure 4.

A, ROCK-dependent Sox9 phosphorylation in TGF-ß-treated SW1353 chondrosarcoma cells. The levels of endogenous Sox9 phosphorylation were assayed by immunoblot in SW1353 cells treated 20 hours with 10ng/ml TGF-$ß_1$. 5μM hydroxyfasudil was added 2 hours prior to and during the TGF-$β_1$ treatment to inhibit ROCK activity. The blot was stripped and re-probed with anti-GAPDH to demonstrate even protein loading. Results shown are typical of 4 independent experiments. **B**, ROCK-dependent increase in nuclear Sox9 in TGF-$ß_1$ treated chondrocytes. Passaged human chondrocytes were grown in alginate suspension culture and treated with TGF-$ß_1$, ROCK inhibitor, or a combination. Cells were fixed and endogenous Sox9 was localized by immunofluorescence microscopy. The amount of nuclear Sox9 was quantified by colocalization with the ToPro3 nuclear dye, shown in arbitrary units on the Y-axis. Error bars indicate the standard deviation of Sox9 nuclear intensity, n=8 cells for each group, * indicates $p<0.01$ compared to control. **C,** Images of an untreated control chondrocyte (left) and a TGF-ß-treated chondrocyte (right) demonstrating increased nuclear localization of Sox9. Nuclei were detected with ToPro3 dye, and Sox9 was detected by anti-Sox9 followed by Alexa488 secondary antibody. Images were taken with a Zeiss LSM510 inverted confocal microscope and the entire selection represents an area of 26um square.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

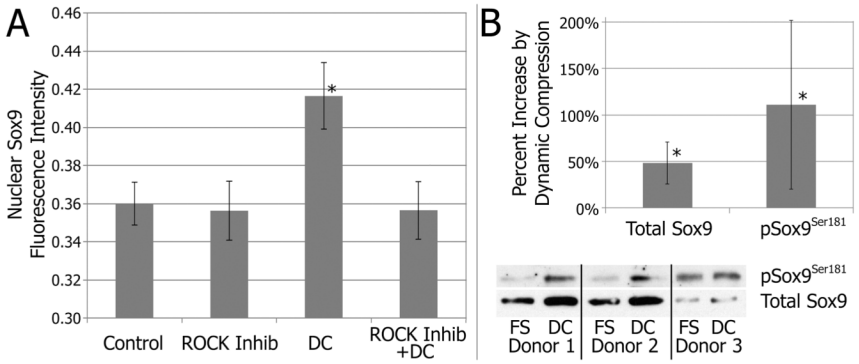

Figure 5.

A, ROCK-dependent increase in nuclear Sox9 after 2 hours of dynamic compression. Alginate-embedded normal human articular chondrocytes were subjected to 2 hours of continuous cyclic dynamic compression from 5–15% strain at 0.5Hz. Cells were fixed, released from alginate, and endogenous Sox9 was quantified by immunofluorescence microscopy. The amount of nuclear Sox9 was quantified by colocalization with the ToPro3 nuclear dye, shown in arbitrary units on the Y-axis. Error bars indicate the 95% confidence interval of the mean, n>300 cells for each group, * indicates $p<0.01$ compared to control. **B**, Increased Sox9 phosphorylation and total protein levels by overnight dynamic compression. Agarose-embedded chondrocytes were subjected to 16–18 hours of dynamic compression (DC), or free-swell uncompressed controls (FS). After dynamic compression, the gels were immediately homogenized in SDS-PAGE sample buffer and analyzed by western blot for the amount of phosphorylated $Sox9^{Ser181}$ with an anti-phospho-$Sox9^{Ser181}$ antibody. Blots were stripped and re-probed for total Sox9 protein levels. The insets graphically show the image densitometry of the immunoblot, error bars indicate the standard deviation from 3 donors.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

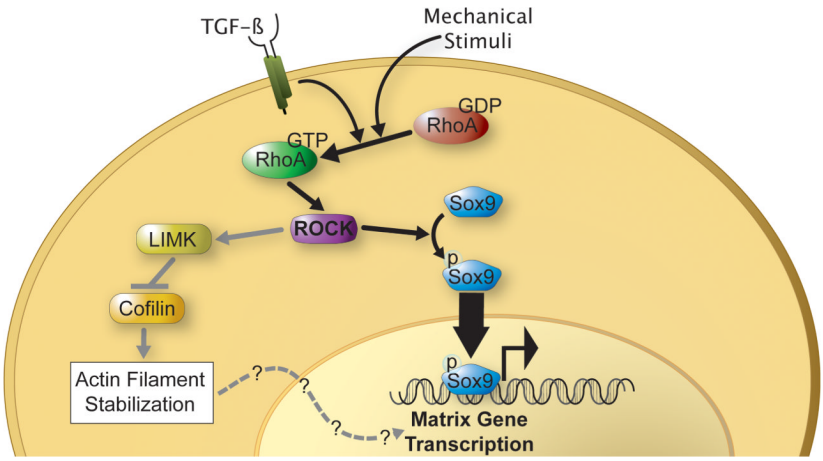

Figure 6. Pathway Schematic

The established pathway of Rho signaling is through ROCK and LIM Kinase to inactivate cofilin, an actin-severing protein. This results in an overall increase in F-actin, which through a series of unknown events leads to changes in matrix gene expression. Based on the observations in this manuscript we present a revised model in which ROCK directly phosphorylates Sox9^{Ser181}, causing increased nuclear localization and enhanced matrix gene expression. Thus, while actin remodeling also occurs upon stimulation of the revised pathway, this is a parallel event to the direct activation of Sox9 by ROCK.