RhoA/ROCK Signaling Regulates Sox9 Expression and Actin Organization during Chondrogenesis

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Abstract

Endochondral ossification is initiated by the differentiation of mesenchymal precursor cells to chondrocytes (chondrogenesis). This process is characterized by a strong interdependence of cell shape, cytoskeletal organization and the onset of chondrogenic gene expression, but the molecular mechanisms mediating these interactions are not known. Here we investigated the role of the RhoA/ROCK pathway, a well-characterized regulator of cytoskeletal organization, in chondrogenesis. We show that pharmacological inhibition of ROCK signaling by Y27632 resulted in increased glycosaminoglycan synthesis and elevated expression of the chondrogenic transcription factor Sox9, whereas overexpression of RhoA in the chondrogenic cell line ATDC5 had the opposite effects. Suppression of Sox9 expression by ROCK signaling was achieved through repression of Sox9 promoter activity. These molecular changes were accompanied by reorganization of the actin cytoskeleton where RhoA/ROCK signaling suppressed cortical actin organization, a hallmark of differentiated chondrocytes. This led us to analyze the regulation of Sox9 expression by drugs affecting cytoskeletal dynamics. Both inhibition of actin polymerization by cytochalasin D and stabilization of existing actin filaments by jasplakinolide resulted in increased Sox9 mRNA levels, whereas inhibition of microtubule polymerization by colchicine completely blocked Sox9 expression. In conclusion, our data suggest that RhoA/ROCK signaling suppresses chondrogenesis through the control of Sox9 expression and actin organization.
Introduction

Chondrocytes fulfill two major roles in mammals. During development, most of our bones form through endochondral ossification in which bones are first laid down as cartilage precursors (1,2). In this process, cartilage serves as a template for subsequent bone formation and controls bone growth through the coordinated proliferation and differentiation of chondrocytes in the growth plate. In the adult, chondrocytes are the sole cell type of articular cartilage and play crucial roles in joint function (3,4). Disruption of chondrocyte function in either context results in severe consequences for affected individuals (4-6). Deregulated proliferation or differentiation of growth plate chondrocytes (e.g. through gene mutations, hormonal disorders or medication) commonly results in skeletal deformities and growth retardation (7,8), whereas loss of articular chondrocyte function is a major contributing factor in the pathogenesis of osteoarthritis (9-11).

Despite some clear differences, growth plate and articular chondrocytes share several common features. Extracellular matrix proteins such as collagen II and aggrecan are among the cartilage markers produced by both cell types (12,13). The transcription factor Sox9 has been shown to be required for chondrocyte formation (chondrogenesis) and directly regulate transcription of the collagen II gene, in conjunction with the related Sox5 and Sox6 genes (14-16).

Another common feature of all chondrocytes is the interdependence of cell shape and differentiation status (17-20). Chondrogenesis is characterized by
drastic changes in cell shape from a fibroblastoid to a round or polygonal morphology (21). This transition is accompanied by changes in gene expression and reverted when chondrocytes dedifferentiate, for example in osteoarthritis and in monolayer culture in vitro (4,22). The molecular mechanisms responsible for this interplay are largely unknown, but the actin cytoskeleton appears to play important roles in this context (23-25). Chondrocytes display mostly cortical organization of their actin filaments in vivo and in vitro, whereas precursor cells or dedifferentiated chondrocytes are characterized by a more fibrillar organization (26-29). Moreover, inhibition of actin polymerization by cytochalasin B has been shown to induce redifferentiation of dedifferentiated chicken chondrocytes (30-34).

These data suggest that the pathways controlling the organization of the chondrocyte actin network could also play major roles in regulating chondrocyte differentiation and function. Rho GTPases are the best characterized upstream regulators of the actin cytoskeleton (35,36). Through a multitude of downstream effectors, they control not only cytoskeletal organization, but also many other cellular functions such as transcription, cell cycle progression and vesicle trafficking (37-39). The kinases ROCKI and ROCKII are among the most important effectors of the prototype GTPase RhoA (40,41). We have shown recently that RhoA/ROCK signaling supports proliferation of established chondrocytes and inhibits hypertrophic differentiation (42), but roles of this pathway in the early steps of chondrogenic differentiation (e.g. the transition from mesenchymal precursor cells to chondrocytes) have not been investigated.
However, important roles of RhoA in the commitment and differentiation of precursor cells into other mesenchymal lineages such as adipocytes, myoblasts and osteoblasts have been reported recently (43,44). This study addresses the roles of RhoA/ROCK signaling and cytoskeletal components in the induction of the chondrogenic phenotype in undifferentiated mesenchymal cells.
Experimental Procedures

Materials

Timed pregnant CD1 mice were purchased from Charles River Laboratories. All cell culture media components were from Invitrogen or Sigma unless stated otherwise. All inhibitors were purchased from Calbiochem or Sigma. All other reagents were of analytical grade from commercial suppliers. The pRlCMV plasmid was from Promega, and the SRF reporter plasmid was purchased from Stratagene. Antibodies against ROCK I (catalogue number Sc-5560) and ROCK II (Sc-5561) were from SantaCruz, the β-actin antibody (A-544) was from Sigma. A plasmid containing the proximal 2 kb of the mouse Sox9 promoter controlling expression of firefly luciferase was provided by Dr. M. Underhill (University of British Columbia).

Micromass culture and ATDC5 cell culture

Mesenchymal limb buds cells were obtained from mice at 11.5 dpc, and cultured in micromass cultures as described (45,46). Briefly, cells were suspended in 60% F12, 40% DMEM, 10% FBS (Gibco), 0.25% PenStrep and 0.25% L-glutamine, at a density of 2.5x10^7 cells/ml and plated in 10 μl droplets to simulate the high density of chondrogenic condensations. One hour after plating, media (as above, supplemented with 1 mM beta-glycerol phosphate and 50 μg/ml of ascorbic acid) was added to the cultures. As indicated, medium is supplemented with DMSO, 10 μM Y27632 (dissolved in DMSO), 3 μM
cytochalasin D (dissolved in DMSO), 50 nM jasplakinolide (dissolved in DMSO),
or 1 µM colchicine (dissolved in water). Media and inhibitors were changed every
24 hours until harvesting.

For confocal microscopy, micromass cultures were plated on glass
coverslips and cultured as above. After one day in culture, cells were fixed in 4%
paraformaldehyde at room temperature for ten minutes, followed by 2 x 10
minutes washes in phosphate buffered saline (PBS). Membranes were then
permeabilized with 0.1% Triton-X/PBS solution for 5 minutes and washed twice
for 5 minutes with PBS. The cells were then incubated in the dark for 50 minutes
at room temperature with 2.5 U/PBS rhodamine phalloidin and mounted with
Vectashield® (Vector Laboratories Inc., Burlingame, CA). Images were taken
using a Zeiss LSM510 Meta confocal microscope with 40-fold magnification and
analyzed using LSM-10 software.

RhoA- and pcDNA3 vector-transfected ATDC5 cells were cultured and induced
to differentiate as described (42).

Reverse Transcriptase and Real-Time PCR

RNA was extracted using the QIAGEN RNeasy kit according to the
manufactures instructions. 500 ng of collected RNA is reverse transcribed using
both random hexamers, pd (N)₆ and poly DT (Amersham Pharmacia). Specific
primers were designed for β-Actin (as described by Stanton et al., 2004), ROCK I
(forward primer; 5'-TTCATGTCCGACCTGTAACC-3' reverse primer; 5'-
TTGACAGCGTTCCAGGAGAG-3') and ROCK II (forward primer; 5'-
TTCACGTCCGACCTGTTACC-3', reverse primer; 5'-GTGGGCACC TACGGCACCCTCTA-3') producing amplicons of 480 and 275 base pairs, respectively. The initial denaturation was done at 95°C for 60-90 seconds, annealing at 65 and 52°C for 30-75 seconds with 30 cycles of amplification for both. Non reverse transcribed RNA served as the negative control. 25 ng of collected RNA was used for real time PCR. Relative gene expression was determined by measuring Sox9 (forward primer; 5'-CATCACCCGCTCGCAATAC-3', reverse primer; 5'-CCGGCTGCGTGACTGTAGTA-3', and probe; 6FAM-5'-ACCATCAGAACTCCGGGCT-MGBNFQ-3 relative to 18srRNA and/or glyceraldehyde-3-phosphate-dehydrogense (GAPDH), using Taqman One step Master Mix and 40 cycles on the ABI Prism 7900 HT sequence detector (Perkin Elmer).

**Western Blot Analysis**

Protein was isolated from treated micromass cultures on days 1 to 4. Cells were collected in cold PBS and centrifuged at 10,000 rpm for 5 minutes at 4°C. The pellets were resuspended in 40 µL of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton-X, 1% deoxycholate, 0.1% SDS, 2 mM EDTA, supplemented with a protease inhibitor mini complete tablet (ROCHE), 50 mM NaF and 1 mM NaVO₃) and stored at -20°C. Samples were then sonicated for 5 seconds at an amplitude of 20% and quantified by BCA (Sigma) as described by the manufacturer’s protocol. Using the BioRad mini-blot apparatus, 40 µg of
total protein was loaded per well with 6x DTT sample buffer and run for 1.5 hours. Protein was transferred onto a nitrocellulose membrane (Schleicher and Schull) and blocked for 2 hours in 5% bovine serum albumin in tris-buffered saline with 0.01% tween-20. One µg/mL of primary antibody of ROCK 1, 2 or actin was incubated overnight at 4°C, followed by incubation with a 5000x dilution of the secondary antibody for 1 hour at room temperature. Signal was detected using ECL™ Western blotting detection reagents (Amersham Bioscience) according to the manufacturer’s protocol and visualized on ChemiImager™5500 (Alphalnnotech Inc).

**Alcian Blue Stain**

Treated micromass cultures were fixed after 2, 3 or 4 days in culture in 100% ethanol for 20 minutes at -20°C and then incubated with 0.1% HCl-Alcian blue for two hours (45). Excess stain was washed off with double distilled water and pictures were taken. Stain was quantified by solubilizing the stain in 6 M guanidine hydrochloride for 8 hours at room temperature. Absorbance was measured using a spectrophotometer at 620 nm. Nodule number was assessed through manual counting by an independent observer unaware of experimental conditions.

**Isolation of primary chondrocytes**

Primary chondrocytes were isolated from tibias of day 15.5 timed mouse embryos. Tibias were isolated and digested for 15 minutes at 37°C with trypsin, followed by a two hour digestion in 3 mg/ml Collagenase P (ROCHE) dissolved in
DMEM (GIBCO) with 10% fetal bovine serum (FBS) (GIBCO). Cells were collected by centrifugation and resuspended in fresh primary culture media (60:40 F12/DMEM + 10% FBS, supplemented with L-glutamine, Pen/Strep). 40,000 cells per well were plated on glass cover slips in a 24 well corning tissue culture plate. After 24 hours, cells are treated with DMSO control or 10 μM Y27632 for 3 hours. Cells were then fixed in 4% paraformaldehyde (at room temperature for ten minutes, followed by 2 x 10 minutes washes in phosphate buffered saline (PBS). Membranes were then permeabilized with 0.1% Triton-X/PBS solution for 5 minutes and washed twice for 5 minutes with PBS. The cells were then incubated in the dark for 50 minutes at room temperature with 2.5 U/PBS rhodamine phalloidin and mounted with Vectashield® + Dapi (Vector Laboratories Inc., Burlingane, CA). Images were taken using a Leica DMRA2 fluorescence microscope with 40-fold magnification and analyzed using OpenLab 3.1 software.

Transfections and Luciferase Assays

Cells for micromass cultures were transiently transfected in suspension prior to plating with a 1:1 ratio of Fugene 6 (ROCHE) and 0.5 μg of a plasmid containing either a serum response factor (SRF) responsive promoter (Stratagene) or the 2 kb proximal promoter of the mouse Sox9 gene linked to the firefly luciferase gene. Cells were always cotransfected with a control plasmid containing the Renilla luciferase gene under the control of the CMV promoter (Promega) to standardize for transfection efficiency. Transfected cells were then
plated in 10 µl droplets as micromass cultures as described above and treated with inhibitors one hour after plating. Three days after transfections, cells were washed with PBS and lysed in lysis buffer (Promega) for 20 minutes at room temperature. 20 µl of lysate were used to determine relative luciferase activity (firefly luciferase activity divided by Renilla luciferase activity) using the Dual Luciferase Assay System (Promega). Data analyses were done as described previously (47).

**Statistical Analysis**

Data collected from Real time PCR are represented as the average of three independent experiments (e.g. three independent isolations of primary cells) run in triplicate. Means were quantified relative to 18S rRNA and/or GAPDH and data were normalized to day 1 of control treated RNA per trial. Alcian blue quantification and nodule counting was an average of three to four independent cell isolations of two replicates per treatment, per time point. Data of luciferase activity represent an average of four independent cell isolations performed in quadruplicate each. Statistical significance was determined with one way ANOVA or two way ANOVA followed by a post-hoc Bonferroni test using GraphPad Prism software.
Results

ROCK I/II are expressed during chondrogenesis

While we had earlier demonstrated expression of ROCKI/II in chondrocytes (42), no data on temporal profiles of their expression during early chondrogenesis are available. We first examined the expression of both ROCK I and II in our micromass cultures. Transcripts were detected with the expected amplicon sizes of 480 and 275 base pairs for ROCKI and II, respectively, throughout the micromass culture period from days 1 to 4 (Fig. 1a). Expression of both kinases was also demonstrated at the protein level by Western blot analyses (Fig. 1b). No obvious changes in ROCKI and II mRNA or protein levels were observed during chondrogenesis.

ROCK suppresses glycosaminoglycan production

We next asked whether inhibition of ROCK signaling would interfere with chondrogenesis by analyzing the effects of the ROCK inhibitor Y27632 (10 μM) on Alcian blue staining. Alcian blue stains for glycosaminoglycans and is therefore an established maker of chondrogenesis. Alcian blue staining increased over time in micromass culture, indicating advanced chondrogenic differentiation. ROCK inhibition did not affect glycosaminoglycan production at days 2 or 3 or micromass culture, but resulted in a visible increase in Alcian blue staining by day 4 of micromass culture (Fig. 2a). Stimulation of glycosaminoglycan production by Y27632 at day 4 was confirmed quantitatively by dye extraction and measurement of absorbance (Fig. 2b). However, the
number of Alcian blue stained nodules at this time point was not affected by Y27632 (Fig. 2c). The size of these nodules also does not appear to change between the control and treated cultures. These data suggest that ROCK inhibition does not affect cell condensation, but results in increased chondrogenic differentiation and chondrocyte-specific extracellular matrix synthesis of mesenchymal precursor cells within nodules.

**ROCK inhibition induces cortical actin morphology**

We next asked whether the effects on glycosaminoglycan synthesis are accompanied by chondrocyte-specific changes in cellular morphology and actin organization. Primary chondrocytes in monolayer culture rapidly lost their rounded morphology and developed a fibroblastoid cell shape with extensive stress fibers (Fig. 3a). Treatment with Y27632 caused reorganization of the actin cytoskeleton to a cortical pattern with parallel rounding of cells, suggesting that ROCK inhibition supports the establishment of a chondrocyte-specific cell shape and actin organization. Similar mechanisms were observed in three-dimensional micromass cultures by confocal microscopy; cultures treated with Y27632 displayed increased cell rounding and a reduced number of actin fibers (Fig. 3b, c).
RhoA overexpression suppresses glycosaminoglycan synthesis and induces stress fiber formation in chondrogenic cells

RhoA is an upstream activator of ROCKI/II and requires ROCK activity for its effects in later stages of chondrogenic differentiation (42). We therefore asked whether RhoA regulates chondrogenesis in a similar fashion as ROCK kinases. Overexpression of RhoA in the chondrogenic cell line ATDC5 resulted in reduced Alcian blue staining (Fig. 4a). These effects were reversed by treatment with Y27632. RhoA overexpression in ATDC5 cells also caused cell elongation and formation of stress fibers, when compared to vector-transfected control cells (Fig. 4b). ROCK inhibition with Y27632 rescued this effect. These data demonstrate that RhoA suppresses chondrogenic differentiation through a ROCKI/II-dependent mechanism.

RhoA/ROCK signaling inhibits Sox9 expression during chondrogenesis

We next asked whether changes in actin organization and glycosaminoglycan expression are accompanied by alteration of chondrogenic gene expression by investigating Sox9 mRNA expression. Similar to Alcian blue staining, Sox9 mRNA levels increased markedly after two days of micromass culture. While Sox9 mRNA levels were similar in Y27632-treated and control cultures until day 2, real-time PCR analysis showed that ROCK inhibition greatly reduces the increase in Sox9 expression on days 3 and 4 (Fig. 5a). RhoA overexpression in the ATDC5 cell line caused a 50% reduction in Sox9 mRNA levels. This effect is rescued by the addition of Y27632 (Fig. 5b). We examined
the effects of Y27632 on the activity of a 2 kb fragment of the mouse Sox9 promoter. ROCK inhibition caused a twofold induction of this promoter fragment in micromass cultures, suggesting that ROCK signaling controls Sox9 mRNA levels through transcriptional mechanisms (Fig. 5c).

**Manipulation of actin polymerization regulates Sox9 mRNA levels**

Our data had shown regulation of chondrocyte actin organization and Sox9 expression by the RhoA/ROCK pathway. Previous studies had demonstrated that inhibition of actin polymerization induces a chondrogenic phenotype (30,31,33), but the molecular mechanism involved had not been described. To clarify whether Sox9 expression is regulated directly by the organization of the actin cytoskeleton, we examined the effects of different drugs that modulate actin remodeling. Cytochalasin D binds monomeric actin and therefore inhibits actin polymerization. In contrast, jasplakinolide binds polymerized actin thereby stabilizing existing filaments and nucleating new actin polymerization. Jasplakinolide treatment caused a significant increase in Sox9 transcript levels at days 3 and 4, whereas cytochalasin D enhanced Sox9 expression by day 4 (Fig. 6A). These data demonstrate that actin dynamics control Sox9 expression and suggest that remodeling of the actin cytoskeleton could contribute to the effects of RhoA/ROCK signaling on chondrogenesis.

Rho GTPases do not only regulate actin organization, but also the microtubule component of the cytoskeleton (48). We investigated the effects of colchicine, an inhibitor of microtubule polymerization, on Sox9 expression in
micromass cultures. Colchicine treatment blocked Sox9 expression at all time points (Fig. 6B), and also completely inhibited Alican blue staining (data not shown). These data show that microtubule polymerization is absolutely required for chondrogenesis to occur, in agreement with earlier studies that have shown reduced glycosaminoglycan and proteoglycan production in colchicine-treated chondrocytes (49,50).

**Effects of cytoskeletal modifications on Serum Response Factor activity**

Previous publications have described a role of the transcription factor serum response factor (SRF) in transcriptional response to cytoskeletal modifications and Rho signaling (51-54). We therefore asked whether the diverse drugs used in this study would signal through SRF. We transiently transfected micromass cultures with an SRF responsive promoter to examine regulation of SRF activity. A significant increase of activity of the SRF is observed upon inhibition of actin or microtubule polymerization (Fig. 7A, B). While Y27632 and Jasplakinolide appeared to activate SRF to some degree, these effects were not statistically significant.
Discussion

The molecular links between cytoskeletal organization and gene expression in chondrocytes are not well understood despite the known relationship of cell shape and differentiation status in these cells and despite the recently discovered roles of actin-regulating pathways in the control of lineage commitment in undifferentiated mesenchymal cells. We hypothesized that pathways regulating actin polymerization control both cell morphology and gene expression during chondrogenic cell differentiation. In this study we show that the RhoA/ROCK pathway indeed fulfills these functions and plays an important role in coordinating actin organization, cell shape and chondrogenic phenotype.

Our data demonstrate that the RhoA/ROCK pathway suppresses glycosaminoglycan synthesis, a marker of early chondrogenic differentiation, without affecting the number or size of the cartilage nodules. This suggests that individual cells within ROCK-inhibited nodules produce more glycosaminoglycans and have progressed further in the chondrogenic program than control cells. These changes in extracellular matrix synthesis are accompanied by parallel changes in actin organization and cell shape. Dedifferentiation of chondrocytes in monolayer culture is characterized by fibroblastoid appearance and formation of stress fibers (18,24). Since stress fiber induction is one of the classical activities of RhoA (55), we postulated that RhoA signaling would suppress the chondrogenic phenotype. Indeed, we demonstrate that pharmacological inhibition of the RhoA/ROCK signaling pathway induces cell rounding and cortical actin organization, hallmarks of differentiated chondrocytes. These effects are seen
both in monolayer cultures of primary, differentiated chondrocytes and during chondrogenesis of mesenchymal precursor cells in three-dimensional micromass cultures. Our pharmacological studies in primary cells are supported by genetic gain-of-function studies in the chondrogenic cell line ATDC5, where RhoA overexpression causes enhanced formation of stress fibers. Although RhoA has been shown to signal through ROCK-independent mechanisms and ROCK activity can be regulated by additional factors (41), our data suggest the effects of RhoA described in this study are mediated by ROCKI/II since Y27632 reverses the effects of RhoA overexpression in ATDC5 cells.

The only transcription factor known to date to be absolutely required for chondrogenesis is Sox9 (14,56,57). This led us to study whether Sox9 expression is affected by RhoA signaling. We show that the RhoA/ROCK pathway controls transcript levels of Sox9 both in primary cells and the chondrogenic cell line ATDC5. RhoA overexpression decreases Sox9 transcripts and inhibition of ROCK in these cells rescues transcripts to control levels. Our data also demonstrate that ROCK inhibition causes upregulation of Sox9 promoter activity, suggesting that the effects of RhoA/ROCK signaling on Sox9 expression are due to transcriptional effects.

Our data show that Y27632 does not affect the number of cartilage nodules, suggesting that the effects of RhoA/ROCK signaling are not due to inhibition of cellular condensation, but rather to delayed cell differentiation within the nodules. In agreement with these data, ROCK inhibition does not affect glycosaminoglycan production and Sox9 expression at early stages of
micromass cultures, but clearly blocks the increase in both parameters at day 4 of differentiation. RhoA/ROCK signaling therefore regulates Sox9 expression, cartilage-specific extracellular matrix synthesis and cell morphology during chondrogenesis.

RhoA/ROCK signaling exerts its cellular effects through cytoskeleton-dependent and –independent mechanisms. We therefore asked whether effects of ROCK inhibition could be mimicked by drugs affecting actin remodeling. Cytochalasin D is an inhibitor of actin polymerization that has been shown promote the chondrogenic phenotype (23,24,34). However, the molecular mechanisms of how disruption of actin polymerization promotes chondrogenesis have not been identified. We show here that cytochalasin D treatment enhances Sox9 expression during chondrogenesis, thus providing a molecular explanation for its chondrogenic activities. Jasplakinolide stabilizes actin filaments and promotes actin polymerization (58) and was therefore expected to have opposing biological activities to cytochalasin D. However, jasplakinolide treatment induced Sox9 mRNA levels even more than cytochalasin D.

While these similar effects of two drugs with apparently opposing biological activities are puzzling, they are not without precedence. For example, both drugs enhance the expression of connective tissue growth factor (59) and inducible nitric oxide synthase (60), activate the transcription factor NFκB (61), suppress expression of VCAM-1 and ICAM-1 (62) and inhibit the activation of cytosolic phospholipase A2 (63). Potential explanations for the common biological effects of cytochalasin D and jasplakinolide are that both block actin
Woods et al. Rho/ROCK suppress chondrogenesis

dynamics and that both reduce the pool of free monomeric actin. Recent studies have shown that levels of monomeric actin directly control the activity of the transcription factor SRF (54,64). SRF is required for mesodermal differentiation (65,66) and has been shown to control chondrocyte gene expression (67), suggesting that reduced levels of monomeric actin in response to cytochalasin D and jasplakinolide could enhance Sox9 transcription through activation of SRF. However, while cytochalasin D strongly activated SRF activity in a reporter gene assay, the effects of Y27632 and jasplakinolide were not significant. Moreover, SRF was also activated by colchicine, despite the observed repression of Sox9 mRNA expression by this compound. SRF activation thus does not appear to correlate with Sox9 expression, making SRF an unlikely mediator of actin-modifying drugs in Sox9 induction.

However, it is quite likely that levels of monomeric actin regulate additional pathways, some of which might be involved in the regulation of Sox9 transcription. Numerous other transcription factors such as NFκB, AP-1 proteins, CREB, STAT5A and GATA factors (39,68-73) have been shown to be regulated by RhoA and/or cytoskeletal dynamics, and are potential candidates for these roles.

Chondrocytes differentiate from precursor cells that also give rise to other mesenchymal lineages such as osteoblasts, myoblasts and adipocytes. Recent data have implicated RhoA signaling in the commitment and differentiation of mesenchymal precursor cells (43,44). In these studies, Rho signaling represses adipogenesis and enhances differentiation along the myogenic and osteogenic
lineages. Our data extend these studies to chondrogenesis and demonstrate that
the RhoA/ROCK pathway inhibits chondrogenesis, highlighting the importance of
Rho GTPase signaling in mesenchymal cell differentiation and lineage
commitment.

In conclusion, we have identified the RhoA/ROCK pathway as an
important regulator of early chondrogenic differentiation that controls cytoskeletal
organization, cell morphology and chondrogenic gene expression. This pathway
therefore appears to play a crucial role in the coordination of different aspects of
chondrogenesis and in the well-established interdependence of cell organization
and differentiation status in chondrocytes. Our data do not only contribute to a
better understanding of the signaling mechanisms controlling mesenchymal and
chondrogenic cell differentiation, but also suggest novel approaches for the
management of musculoskeletal diseases characterized by insufficient growth or
loss of cartilage, such as chondrodysplasias and osteoarthritis.
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Footnotes

1, abbreviations: PBS, phosphate-buffered saline; SRF, serum response factor
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Figure Legends

Figure 1: ROCK I/II are expressed during chondrogenesis

a) RT-PCR analyses show that transcripts of both ROCK I (480 basepair amplicon) and II (275 basepair amplicon) are expressed throughout the time course studied in micromass culture of days 1 to 4. β-Actin (605 basepair amplicon) was used as a loading control. Negative controls (reaction in the absence of reverse transcriptase) produced no amplicons (data not shown).

b) Western blot analysis of proteins isolated from days 1 to 4 of micromass culture show that ROCK I and II proteins are expressed throughout the time course. Probing for β-actin demonstrates equal loading and transfer.

Figure 2: ROCK signaling inhibits glycosaminoglycan synthesis

Micromass cultures treated daily with 10 µM Y27632 or vehicle were stained with Alcian blue to visualize glycosaminoglycans on days 2, 3 and 4 of culture (a). After solubilization in 6M guanidine hydrochloride, stain taken up by micromasses was quantified by measuring absorbance of 620 nm (b). The number of Alcian blue-stained nodules of day 4 micromass cultures was counted manually (c). Data shown in b and c represent average and SEM from at least three independent experiments run in duplicate each. Y27632 treatment causes a significant increase in total glycosaminoglycan synthesis, but does not affect number or size of cartilage nodules (* p < 0.05).
Figure 3: ROCK inhibition causes cortical actin organization

(a) Primary chondrocytes were plated in monolayer culture for 24 hours followed by 3 hour treatment with DMSO (control) or 10 µM Y27632. Cells were then fixed in paraformaldehyde and stained with rhodamine-phalloidin (red) for the actin cytoskeleton and Hoechst for nuclei (blue). Representative cells are shown. Monolayer culture induces cell spreading and stress fiber formation in control cells, whereas ROCK inhibition results in cell rounding and cortical actin organization, characteristic features of chondrocytes.

(b, c) Micromass cultures were plated for 1 day in culture and treated with vehicle or Y27632. Cells were then fixed in paraformaldehyde and stained with rhodamine-phalloidin for the actin cytoskeleton. Representative cells are shown. In threedimensional culture, ROCK inhibition results in greater numbers of cell rounding and a reduced number of actin fibers, characteristic features of chondrocytes.

Figure 4: RhoA overexpression inhibits glycosaminoglycan synthesis and enhances stress fiber formation

a) ATDC5 cells stably transfected with RhoA or vector control were induced to differentiate with ITS and cultured for 15 days in the presence of vehicle or 10 µM Y27632 before staining with Alcian blue. RhoA overexpression suppresses glycosaminoglycan synthesis, but ROCK inhibition reverses this effect.

b) ATDC5 cells stably transfected with RhoA or vector control were induced to differentiate with ITS and cultured for 6 days in the presence of vehicle or 10 µM
Y27632 The cultures were fixed and stained with rhodamine-phalloidin (red) for the actin cytoskeleton and Hoechst for the nucleus (blue). RhoA overexpression causes increased stress fiber formation, an effect that is reversed upon inhibition of ROCK.

**Figure 5: The RhoA/ROCK pathway inhibits Sox9 expression**

a) Sox9 mRNA levels at days 1, 2, 3 and 4 of micromass cultures treated with vehicle or 10 µM Y27632 were analyzed by real-time PCR. ROCK inhibition results in a twofold increase in Sox9 mRNA levels at days 3 and 4.

b) Sox9 mRNA levels in ATDC5 cells stably transfected with RhoA or vector and treated with vehicle or 10 µM Y27632 were analyzed by real-time PCR at days 3 and 6 of differentiation. RhoA overexpression reduces Sox9 mRNA levels by day 6, but ROCK inhibition rescues this effect.

All data shown are the mean relative gene expression ± standard error from three independent trials run in triplicate each normalized to 18s rRNA and/or GAPDH RNA levels (*: p< 0.05).

c) Micromass cultures were transiently transfected with a 2 kb minimal Sox9 promoter construct and pRICMV (for normalization) and treated daily with vehicle or 10 µM Y27632. After 3 days, cells were harvested and relative luciferase activity was determined by normalizing firefly luciferase activity to renilla luciferase activity. The Sox9 promoter conferred a two-fold increase in luciferase activity when the RhoA/ROCK pathway was inhibited. Data shown are an
average of relative luciferase activity of four independent experiments run in quadruplicate ± standard error (*: p< 0.05).

**Figure 6: Effects of cytoskeletal drugs on Sox9 expression**

a) Micromass cultures were incubated in the presence of vehicle, cytochalasin D (3 μM) or jasplakinolide (50 nM) for 1, 2, 3 and 4 days. Sox9 mRNA levels were analyzed by real-time PCR. Both treatments resulted in increased Sox9 mRNA levels at day 4, but jasplakinolide also caused significant increases in Sox9 expression at day 3.

b) Micromass cultures were incubated in the absence (control) or presence of colchicine (1 μM) for 1 to 4 days. Sox9 mRNA levels were analyzed by real-time PCR. Colchicine treatment strongly inhibits Sox9 expression at all time points. Data shown are the mean relative gene expression ± standard error from three independent experiments, run in triplicate each and normalized to 18s rRNA and/or GAPDH RNA levels (*: p< 0.05).

**Figure 7: Disruption of actin or microtubule polymerization increases SRF activity**

Micromass cultures were transiently transfected with an SRF responsive promoter construct and pRlCMV (for normalization) and treated daily with a) DMSO, 10 μM Y27632, 3 μM Cytochalasin D, or 50 nM Jasplakinolide; or b) No treatment or 1 μM Colchicine. After 3 days, cells were harvested, firefly luciferase activity was determined and normalized to Renilla luciferase activity to yield
relative luciferase activity. Cytochalasin D and colchicine treatment result in significant activation of SRF. Data shown are mean and standard error of three independent trials done in quadruplicate each (*: p<0.05 ).
Figure 1

a) Days

|   | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| ROCK I | | | | |
| ROCK II | | | | |
| Actin | | | | |

b) Days

|   | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| ROCK I | | | | |
| ROCK II | | | | |
| Actin | | | | |
Figure 2

a) Vehicle

Day 2
Day 3
Day 4

Y27632

b) Absorbance 620 nm

Condensations/micromass

Day 4
Figure 3

Vehicle       Y27632

a)            

b)            

10 µm

c)            

5 µm
Figure 4

a) Vector  RhoA  RhoA + Y27632

250 mm

b) Vector  RhoA  RhoA + Y27632

10 μm
Figure 5

a) Relative Gene Expression over Days

- Vehicle
- Y27632

b) Relative Gene Expression over Days

- Vector
- RhoA
- RhoA + Y27632

c) Relative Light Units over Day 3

- Vehicle
- Y27632
Figure 6

(a) Relative Gene Expression over days for Vehicle, Cytochalasin D, and Jasplakinolide.

(b) Relative Gene Expression over days for Vehicle and Colchicine.
Figure 7

(a) Graph showing Relative Light Units for Vehicle, Y27632, Cytochalasin D, and Jasplakinolide.

(b) Graph showing Relative Light Units for Vehicle and Colchicine.
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