Rho Kinase Phosphorylates and Activates Sox9 in Chondrocytes

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INTRODUCTION:
Sox9 is a transcription factor that directly regulates the formation of cartilage extracellular matrix during embryogenesis. Sox9 enhances the transcription of collagens type II, IX, and XI, which together form the major collagen fibrils within cartilage. Sox9 also controls the production of aggrecan and link protein, which together with hyaluronan form the major proteoglycan component in cartilage matrix. Sox9 subsequently helps maintain the chondrocyte phenotype by inhibiting the progression toward hypertrophy in proliferating chondrocytes.

The activity of Sox9 is regulated by posttranslational modifications, which occur at several levels. Ubiquitination and sumoylation sites have been identified; however, phosphorylation is by far the most widely studied modification of Sox9. Sox9 contains two sites for phosphorylation by PKA at Serine 64 and Serine 181. Phosphorylation of Sox9 at these sites by PKA can lead to increased DNA binding and transcriptional activity. Sox9 181 is located next to a nuclear localization signal in Sox9.

Chondrocyte cell shape is dependent largely on the conformation of the actin cytoskeleton, and cell shape is linked to chondrocyte phenotype and differentiation status. Disruption of the actin cytoskeleton in monolayer cultures causes cell rounding and altered cartilage matrix production. Rho Kinase (ROCK) plays a central role in the actin dynamics and therefore also dramatically affects chondrocyte cell shape.

ROCK activity and the actin cytoskeleton have recently been correlated to chondrocyte gene expression but without a proposed mechanism. In this abstract, we first demonstrate that ROCK directly phosphorylates Sox9 to increase its nuclear localization and transcriptional activity. We also show that this pathway is activated by two separate anabolic stimuli: TGF-beta and physiologic mechanical compression.

METHODS:
Plasmid Transfections: SW1353 human chondrosarcoma cells were seeded onto 6cm tissue-culture dishes at a density of 4.5x10^5 cells/cm^2 and allowed to attach overnight. Cells were then transfected with Lipofectamine 2000.

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.

Sox9 Transcriptional Activation Luciferase Assay: We used a luciferase construct driven by a 480bp Col2a1 intron 1 enhancer element known to be activated by Sox9. Constant amounts of luciferase reporter (150ng), renilla control (15ng) and Sox9-FLAG (150pg) plasmids were co-transfected with various amounts of ROCK plasmids. The total amount of transfected DNA was held constant by adding empty vector where necessary. Cells were lysed and luciferase and renilla activities were analyzed using the Dual-Luciferase reporter assay system (Promega, Madison WI).

Sox9 Nuclear Localization: Algninate-embedded chondrocytes were treated with dynamic compression (2 hours of 5-15% strain at 0.5 Hz) or 10ng/ml TGF-beta for 20 hours. Cells were fixed, released from alginate, gently spun onto a glass slide, and processed for immunofluorescence using an anti-Sox9. ToPro3 dye was used to identify the nuclei. Images were collected on a Zeiss LSM510 microscope with a 63x water immersion lens. Colocalization of Sox9 and nuclei was performed with custom software algorithms.

RESULTS:
We identified a potential Rho Kinase consensus sequence at Serine 181 of Sox9 (Fig 1A), and using purified proteins in vitro, demonstrated that a direct interaction occurs between ROCK and Sox9 in which Serine 181 of Sox9 is a target for phosphorylation by ROCK (Fig 1B).

To test for intracellular interaction between ROCK and Sox9, we transfected SW1353 cells with plasmids coding for Sox9 and ROCK, and analyzed the extent of Sox9 phosphorylation by western blotting with a phospho-specific antibody. A kinase-dead (KD) ROCK mutant was a negative control (Fig 1C).

DISCUSSION:
Chondrocyte gene expression is greatly affected by cell shape, which in turn is controlled by the actin cytoskeleton. Sox9 is a master regulator of chondrocyte gene expression, and ROCK is a major pathway regulating cell shape via the actin cytoskeleton. To clarify the mechanisms linking these observations, we tested the hypothesis that Sox9 is a direct target for phosphorylation by ROCK. We demonstrated that ROCK does phosphorylate Sox9 both in vitro and in a cellular context, and that this leads to increased nuclear accumulation of Sox9 upon anabolic stimulation. We further showed that ROCK enhances the transcriptional activity of Sox9 using a luciferase reporter assay. Together, these results help provide a molecular mechanism to explain how actin reorganization through the known activities of Rho Kinase might be functionally linked to altered gene expression via the newly identified ROCK-Sox9 interaction.