Introduction: Recent studies indicate that Notch signaling is involved in repressing mesenchymal stem cell (MSC) chondrogenic differentiation (1,2), and we previously demonstrated that TGFβ3 is required to modulate Notch's repressive influence to allow chondrogenesis (3). However, the mechanism of this repression and how this is modulated to allow chondrogenic differentiation has not been fully elucidated. In this study we focused on the potential role of Notch signaling proteins, hairy and enhancer of split 1 (Hes-1) and hairy and enhancer of split related-2 (HERP-2/Hey-1) in repressing chondrogenic differentiation via over expression studies. Further, since Hes-1 and Hey-1 are known to repress transcription by binding to DNA and recruiting repressor proteins, we specifically examined their potential to bind to putative N-Box binding domains located in the enhancer site of Col2a1.

Materials and Methods: Human Tissue Samples. Human bone marrow derived mesenchymal stem cells (hMSC) were isolated from the iliac crest bone marrow of normal adult donors (approval by human subjects committee). Pellet cultures. hMSC pellets were cultured in 0.5 ml chondrogenic medium (Cambrex), supplemented with BMP6 (500 ng/ml) and TGFβ3 (10 ng/ml). Over Expression of NICD, Hes-1 and Hey-1. hMSC were transfected with either 1µg of NICD-pCDNA3.1, pcMV-FLAG-Hes-1, pcDNA3-FLAG-Hey-1, or pcDNA3.1 in monolayer culture using FUGENE HD (Roche). Pellet cultures were made after 2 days. RNA Extraction and Quantitative PCR. Total RNA was extracted from each pellet after 6 days using the RNeasy kit (Qiagen) for detection of Col2a1, aggrecan, Sox9 and GAPDH. Chromatin Immunoprecipitation (chIP) Assays. For each chIP assay 1x106 cells were used. Nuclear extracts were re-suspended in high salt buffer (Santa Cruz) and sonicated. The chromatin lysate were incubated with either anti-Hes-1 (Santa Cruz), anti-Hey-1 (Avia Systems) or the species-matched IgG. Protein A/G Agarose beads (Santa Cruz) were added to each tube and incubated for 2 h at 4°C. DNA was isolated using the QuickExtract gel extraction kit (Qiagen) and PCR was then performed using a specific primer set designed to amplify putative N-Box regions located in intron 1 of the Col2a1 enhancer sequence.

Results: NICD, Hes-1 and Hey-1 Over Expression Repress Chondrogenic Differentiation. NICD and Hes-1 overexpression led to a 2- to 3-fold repression in Col2a1 transcription relative to the pcDNA controls. A surprisingly strong (approximately 80-fold) repression was found in Hey-1 transfected pellet cultures (Fig. 1). NICD and Hes-1 did not alter aggrecan transcription, yet Hey-1 showed a 10-fold repression (Fig. 1). Sox9 expression was not altered following any of these treatments. Hes-1 and Hey-1 Bind to N-box Domains Located in Intron 1 of Col2a1. We located two putative N-Box binding sites, one -190bp (CACGAG) from the Sox9 binding site. Since Sox9 binding to this region is essential for transcriptional activation of Col2a1, it is likely that Hes-1 and Hey-1 compete with Sox9 for binding to this site and hence mediate transcriptional activity. Aggrecan expression is also mediated by Sox9 (5) and Hey-1 over expression strongly repressed its expression. Consistently, we have located putative N-box domains in several domains in intron 1 of aggrecan. Together these results reveal the mechanism by which Notch signaling represses chondrogenic differentiation.

Discussion: Our data confirms, in hMSC, that NICD over expression suppresses chondrocytic differentiation. Both Hes-1 and NICD over expression lead to a decrease in Col2a1 expression, but Hey-1 had the strongest repressive effect. Our chIP assays reveal that both Hes-1 and Hey-1 bind to N-box domains located in the Col2a1, one of which is identical to a known Sox9 binding site. Since Sox9 binding to this region is essential for transcriptional activation of Col2a1, it is likely that Hes-1 and Hey-1 compete with Sox9 for binding to this site and hence mediate transcriptional activity. Aggrecan expression is also mediated by Sox9 (5) and Hey-1 over expression strongly repressed its expression. Consistently, we have located putative N-box domains in several domains in intron 1 of aggrecan. Together these results reveal the mechanism by which Notch signaling represses chondrogenic differentiation.

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