Novel Functions for Runx Proteins During Chondrocyte Maturation

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Introduction: Runx proteins are cell fate determining transcription factors. Runx1 is essential for hematopoietic differentiation, Runx2 is required for osteogenesis, and Runx3 is involved in both neurologic and gut development. All three Runx factors are expressed during chondrocyte differentiation. Runx1 is found in proliferating chondrocytes, while Runx2 and Runx3 are found in hypertrophic chondrocytes.1 During chondrogenesis, Runx2 controls genes required for hypertrophy that include Collagen10a1, VEGF, and MMP13. In the absence of Runx2 in vivo, only chondrocytes in the most distal limbs reach hypertrophy. In the absence of Runx2 and Runx3, there are no hypertrophic chondrocytes found in the skeleton.1 It appears that Runx1, Runx2, and Runx3 each have distinct roles during chondrogenesis.

Studies in bone demonstrate that Runx2 epigenetically regulates genes by remaining bound to target loci throughout mitosis, poised them in a transcriptionally active state.2 Runx2 also regulates protein synthesis in the osteoblast, by controlling ribosomal gene synthesis.2 Similar studies have shown that tissue specific transcription factors, MyoD in myocytes, C/EBPα in adipocytes, and Runx3 in cancer cells, remain associated with target genes throughout the cell cycle, without affecting the transcription of genes, but rather contributing to epigenetic regulation.3,4

In this study we examined which of the Runx factors carried out these novel functions in chondrocytes during chondrogenesis from the mesenchymal stem cell to the hypertrophic stage. Our findings provide direct evidence that: 1) Runx2 functions epigenetically by remaining bound to chromosomes during mitosis; 2) in proliferating chondrocyte precursors, Runx1 regulates protein synthesis by controlling expression of pre-rRNA; and 3) Runx2 binds to the promoters of ribosomal DNA repeats, indicating a role in control of protein synthesis during chondrocyte hypertrophy.

Materials and Methods: Two cell culture models were used, representing different stages of chondrogenesis. N1511 cells, that represent committed chondrocytes that differentiate into hypertrophic chondrocytes, were cultured in Alpha-MEM containing 10% FBS where they were maintained as chondrogenic precursors. Colcemid was added to the cultures at a concentration of 30ng/mL, for 16 hours to block the cells in metaphase. Cells were harvested by mitotic shake off, fixed in 3.5% formaldehyde, and spun onto slides. Immunofluorescence was performed on the metaphase spreads using the Runx2 M70 antibody (Santa Cruz).

ATDC5 cells represent pre-chondrocytes when cultured in DMEM/F-12 containing 10% FBS, transferrin, and sodium selenite. To induce differentiation, insulin was added to the media. Media was changed every other day and cells were harvested at day 21 for chromatin immunoprecipitation experiments.

To address Runx1 functions at the onset of chondrogenesis, Runx1 overexpression was performed in ATDC5 cells plated at a density of 100,000 cells/well in a 6-well plate. After 24 hours, cells were transfected with a CMV-Runx1 vector using Fugene transfection reagent (Promega). Cells were harvested for RNA at 48 hours. RNA was reverse transcribed into cDNA and subjected to qPCR analysis.

Results: Although Runx1 is highly expressed in ATDC5 and N1511 cells, it was not found to be associated with mitotic chromosomes. However, N1511 chondrogenic cells expressing high levels of Runx2, when synchronized in metaphase using Colcemid and subjected to immunofluorescence analysis, show that Runx2 is found in distinct foci on metaphase chromosomes (Figure 1), indicating involvement in epigenetic regulation of target genes in this cell line.

To determine if Runx1 influenced global protein synthesis in proliferating chondrocytes, ATDC5 cells were cultured as proliferating chondrogenic precursors. Runx1 was overexpressed, and RNA was isolated 48 hours after transfection to determine levels of protein synthesis machinery. Quantitative real time PCR analysis shows that, in cells overexpressing Runx1, levels of pre-rRNA are three times higher than in empty vector controls. This result indicates that Runx1 upregulates global protein synthesis in proliferating chondrocytes. Because expression of Runx1 decreases and expression of Runx2 increases as chondrocytes enter the hypertrophic stage, we hypothesized that Runx2 may regulate global protein synthesis at later stages of chondrogenesis. To test this theory, we plated ATDC5 cells under differentiation conditions. At day 21, when levels of Runx2 begin to increase, we harvested cells for chromatin immunoprecipitation assays (ChIP). In these differentiated cells, we found that Runx2 occupies sites in the promoters of ribosomal DNA repeats, indicating direct control of protein synthesis in hypertrophic chondrocytes.

Discussion: We identified novel roles for Runx1 and Runx2 during chondrogenesis. Runx2 has long been known to play an important role during chondrocyte hypertrophy. Many of the genes that are expressed in hypertrophic chondrocytes are direct targets of Runx2. Here, we show that Runx2 remains bound to chromosomes throughout mitosis, indicating that it is epigenetically regulating target genes during chondrogenesis. Because low levels of Runx2 are present in mesenchymal progenitor cells, it is possible that Runx2 is “bookmarking” genes that are essential for chondrocyte hypertrophy in early chondroprogenitor cells, although these genes are not expressed until later in chondrocyte maturation.

We identify possible mechanisms of protein synthesis control during chondrogenesis. We show that overexpression of Runx1 in chondroprogenitors increases levels of pre-rRNA, which is an important component of the protein synthesis machinery. Other studies from our lab demonstrate that, in osteoarthritis (OA), Runx1 is associated with clonal populations of chondrocytes that are characteristic of advanced OA.5 These clonal populations of chondrocytes also express PCNA, indicating that they are proliferative. It is possible that in proliferating chondrocytes of the growth plate, as well as in clonal populations of chondrocytes in OA, mediates an increase in the machinery for protein synthesis to support the capacity for proliferation.

During later stages of chondrogenesis, we observe that Runx2 may assume control of protein synthesis. We find that Runx2 occupies the promoters of ribosomal DNA repeats in hypertrophic chondrocytes. We also provide evidence that, during chondrogenesis, and Runx2 exhibits reciprocal suppression. Consistent with this finding, such cross-regulation may account for the variation in protein synthesis that occurs when proliferating chondrocytes transition to hypertrophic chondrocytes. Understanding mechanisms that control protein synthesis and regulation of target genes can contribute to understanding differences between populations of chondrocytes, both in the growth plate and in articular cartilage. Defining properties of chondrocyte populations will support the identification of specific subpopulations that can preferentially regenerate damaged cartilage, providing a novel basis for advances in cartilage repair and osteoarthritis therapy.

Figure 1. Functions of Runx Factors in Chondrocytes. Left – Epigenetic control. Foci of Runx2 (green) bound on metaphase chromosomes (blue) in N1511 cells. Right – Protein synthesis. Overexpression of Runx1 in ATDC5 cells results in increased levels of pre-rRNA.

References:
5. LeBlanc, K; et al. ORS 2010

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