# SERPINA3 (ALPHA-1 ANTICHYMOTRYPSIN) IS ESSENTIAL FOR EXTRACELLULAR MATRIX PRODUCTION DURING CARTILAGE FORMATION AND REGULATES THE CHONDROGENIC TRANSCRIPTION FACTOR SOX9: IMPLICATIONS FOR CARTILAGE DEVELOPMENT AND REGENERATION

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Purpose

The developing skeleton begins as cartilage, formed from condensation of early mesenchymal tissue which undergo chondrogenesis. This process can readily be modelled *in vitro*, by culturing mesenchymal stem cells (MSCs) at high density in a defined medium. Serine proteinase inhibitors (serpins) are a superfamily of structurally related proteins, the majority of which are inhibitory. We have previously outlined the emerging role of (serpins) in cartilage biology and homeostasis. Here, we aimed to evaluate their role in cartilage development, and we identified an essential non-redundant role for one serpin, SERPINA3 (alpha-1 antichymotrypsin), in chondrogenesis.

Methods

MSCs were differentiated into chondrocytes by culturing at high density in chondrogenic medium. Serpin expression was determined by analysis of a previously published chondrogenesis transcriptome dataset (GSE109503). *SERPINA3* and chondrogenic markers gene expression was determined by real-time PCR using RNA extracted from *in vitro* chondrogenesis, or from developing human tissues. Immunohistochemistry was performed to determine SERPINA3 protein localisation in cartilage pellets, while immunofluorescence and confocal microscopy was used to establish SERPINA3 intracellular expression in adult human articular chondrocytes (HAC). Depletion of SERPINA3 was performed by transfecting small interfering (si)RNA into MSCs prior to chondrogenic differentiation. Sulphated glycosaminoglycan (GAG) levels were measured as a surrogate for proteoglycan and determined histologically by safranin-O staining, or biochemically using the dimethyl-methylene blue (DMMB) assay. Bulk RNA sequencing (RNA-seq) of chondrogenic and osteogenic differentiation was performed following siRNA silencing of SERPINA3, at Days 0, 3 and 7. Changes in SOX9 protein levels following depletion of SERPINA3 was determined at early timepoints (Day 0, 1 and 2) by western blotting.

Results

*SERPINA3* expression was induced early and robustly during chondrogenesis (74-fold, day 1). SERPINA3 in cartilage pellets was notably cellular, and in adult chondrocytes confocal microscopy revealed punctate cytoplasmic and nuclear staining. In developing human tissues *SERPINA3* expression was enriched in cartilage compared to whole limb RNA ($p<0.001$), and correlated with other chondrogenic markers. Depletion of SERPINA3 using siRNA was robust, and expression remained significantly reduced over 14 days. Loss of SERPINA3 led to smaller cartilage pellet formation, with significantly reduced GAG content ($p<0.001$). When comparing siSERPINA3 to a non-targeting control (siControl), bulk RNA sequencing revealed 476 genes were significantly differentially regulated at day 7 (123 upregulated, 353 downregulated; fold change >2, $p_{adj}<0.01$). Induction of several cartilage marker genes were all markedly downregulated, including *COL2A1* ($P_{adj}=0.0004$), *ACAN* ($P_{adj}=0.0002$), *MATN3* $P_{adj}<0.0001$) and pathway analysis supported significant differences in pathways affecting the extracellular matrix. RNAseq following osteogenesis suggested that the effect was specific to chondrogenic differentiation. Due to the global effect on chondrogenesis, we investigated the effect of SERPINA3 depletion on SOX9 protein induction, which was abrogated at early time points.

Conclusions

SERPINA3 is induced early in chondrogenesis and plays an essential, non-redundant role in chondrogenic differentiation. SERPINA3 depletion abrogated chondrogenesis, leading to reduced pellet size, GAG content and expression of cartilage marker genes. The negligible effect of SERPINA3 loss in osteogenesis, supports a role for SERPINA3 specific to MSC differentiation into chondrocytes. Loss of SERPINA3 blocked the protein induction of SOX9, the master transcriptional regulator of chondrogenesis. We hypothesise that SERPINA3 plays an important intracellular role in the regulation SOX9-driven chondrogenesis, although this requires further investigation. Our study has implications for the molecular mechanisms of early skeletal development and for cartilage regenerative medicine strategies.