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Hmgb1 can facilitate activation of the matrilin-1 gene promoter by Sox9 and L-Sox5/Sox6 in early steps of chondrogenesis

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ABSTRACT

The architectural high mobility group box 1 (Hmgb1) protein acts as both a nuclear and an extracellular regulator of various biological processes, including skeletogenesis. Here we report its contribution to the evolutionarily conserved, distinctive regulation of the matrilin-1 gene (*Matn1*) expression in amniotes. We previously demonstrated that uniquely assembled proximal promoter elements restrict *Matn1* expression to specific growth plate cartilage zones by allowing varying doses of L-Sox5/Sox6 and Nfi proteins to fine-tune their Sox9-mediated transactivation. Here, we dissected the regulatory mechanisms underlying the activity of a conserved distal promoter element 1. We show that this element carries three Sox-binding sites, works as an enhancer *in vivo*, and allows promoter activation by the Sox5/6/9 chondrogenic trio. In early steps of chondrogenesis, declining *Hmgb1* expression overlaps with the onset of *Sox9* expression. Unlike repression in late steps, Hmgb1 overexpression in early chondrogenesis increases *Matn1* promoter activation by the Sox trio, and forced Hmgb1 expression in COS-7 cells facilitates induction of *Matn1* expression by the Sox trio. The conserved *Matn1* control elements bind Hmgb1 and SOX9 with opposite efficiency *in vitro*. They show higher HMGB1 than SOX trio occupancy in established chondrogenic cell lines, and HMGB1 silencing greatly increases *MATN1* and *COL2A1* expression. Together, these data thus suggest a model whereby Hmgb1 helps recruit the Sox trio to the *Matn1* promoter and thereby facilitates activation of the gene in early chondrogenesis. We anticipate that Hmgb1 may similarly affect transcription of other cartilage-specific genes.

1. Introduction

SRY-related high-mobility-group (HMG) box (Sox) proteins and canonical high-mobility-group box (HMGB) proteins have distantly related DNA-binding domains that regulate gene expression by diverse mechanisms during development and disease. Several Sox proteins control cell-fate decisions in early steps of endochondral bone formation [1], whereas the Hmgb1 protein was shown to regulate later steps [2]. We determined here the role of Hmgb1 in early steps by focusing on the control region of the matrilin-1 gene (*Matn1*) [3].

The Sox and Hmgb protein families have similar as well as distinct features [1,4,5]. Their HMG boxes show only 20% identity, but both interact with the minor groove of the DNA helix and induce a sharp bend of this helix upon binding. While Hmgb proteins are abundant and widely expressed non-histone chromatin components, Sox proteins are expressed at a low level and only in certain cell types. Hmgb proteins bind distorted DNA transiently and without sequence specificity [4,6]. Lacking a transactivation domain, they act only as architectural

Abbreviations: CEC, chicken embryo chondrocyte; CEF, chicken embryo fibroblast; ChIP, chromatin immunoprecipitation; Dpe1 and Dpe2, distal promoter upstream elements 1 and 2; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; GP, growth plate; GST, glutathione S-transferase; HDM, high density mesenchyme; HMG, high-mobility-group; Hmgb, HMG box; Ine, initiator element; LDM, low density mesenchyme; LM-PCR, ligation-mediated PCR; Nfi, nuclear factor I; Pe1, promoter element 1; RCS, rat chondrosarcoma; SI and SII, silencer elements I and II

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proteins to alter the chromatin structure and modulate transcription. Hmgb1 can promote transcription by displacing the histone H1 and thereby increasing the accessibility of chromosomal DNA to regulatory factors [7,8]. By bending DNA, it helps recruit general transcription factors to the promoter and, *via* protein–protein interactions, it can enhance the binding of classical transcription factors to their cognate sites and promote the recruitment of additional interacting factors [4]. By contrast, Sox proteins bind DNA specifically, although with lower sequence specificity and efficiency than classical transcription factors [5]. In addition to a postulated architectural function, some Sox proteins (*e.g.* Sox9) feature a potent transactivation domain, and thereby act as classical transcription factors. They interact with many partner factors to facilitate enhanceosome formation and control cell fate and differentiation during development [1]. The Sox9, L-Sox5 and Sox6 proteins are referred to as chondrogenic Sox trio, as they act cooperatively to direct the lineage commitment and differentiation of chondrocytes during endochondral bone development.

Endochondral ossification is the process whereby vertebrate embryos first form cartilaginous skeletal structures and then remodel them into bone and bone marrow tissue. It involves strict spatial and temporal coordination of morphogenetic and differentiation steps [9–11]. First chondrogenesis takes place, which starts with the condensation of mesenchymal precursors and their differentiation into chondroblasts that proliferate and deposit an extensive cartilaginous extracellular matrix (ECM) [10,12,13]. This matrix is primarily made of collagen-2 (encoded by *Col2a1*) and aggrecan (*Acan*), which determine its physical properties, while matrilins and other minor components modulate the ECM organization. Then, in the middle of cartilaginous primordia, proliferative chondroblasts exit from the cell cycle, differentiate into prehypertrophic and hypertrophic cells, and eventually undergo apoptosis. Blood vessels, osteoclasts and osteoblasts then invade and replace the mineralized hypertrophic cartilage by bone tissue. This sequential differentiation of chondrocytes results in the formation of distinct tissue zones, collectively referred to as the growth plate (GP), in line with their essential role in skeletal elongation.

The multi-step chondrocyte differentiation process is marked by sequential changes in gene expression [12,14,15]. *Col2a1* starts to be expressed in prechondrocytes, whereas *Acan* and most other cartilage ECM genes are turned on in early chondroblasts and upregulated in the columnar or proliferating GP zone. By contrast, *Matn1* shows such a slow and delayed activation in early chondroblasts [15–18] that is expressed almost exclusively in columnar and prehypertrophic chondrocytes [19–21]. Sox9 directs the commitment of osteochondroprogenitors to the chondrocyte lineage [22,23], and together with L-Sox5 and Sox6, directly controls the subsequent steps of chondrocyte differentiation [24–26]. The Sox trio namely activates *Col2a1*, *Acan* and most other cartilage ECM genes [12,22,27,28]. Sox9 binds as a homodimer to inverted pairs of Sox motifs in cartilage-specific enhancers to activate transcription *via* its transactivation domain. L-Sox5/Sox6 binds more variable Sox motifs. Lacking a transactivation domain, they synergize with Sox9 by increasing its binding efficiency [29]. Various patterning factors, signaling molecules (*e.g.* Ihh/PTHrP, FGF, BMP), and hormones influence the shape and size of skeletal elements and bone growth [10–12,30,31]. Endochondral ossification is defective in *Hmgb1*$^{-/-}$ mice, as Hmgb1 is secreted by hypertrophic chondrocytes and acts as a chemoattractant for cell invasion [2].

Matrilins are multidomain filament-forming proteins, which serve as adaptors in ECM assembly and in mechanotransduction of chondrocytes [32–34]. Unlike other matrilins, *Matn1* is expressed exclusively in cartilage, where it mediates connections between aggrecan, collagen-2 and other molecules. Pericellular Matn1 and Matn3 are needed for Ihh signaling and mechanical stimulation of chondrocyte proliferation and differentiation [35]. *MATN1* polymorphism has been linked to mandibular prognatism in human and *Matn1* upregulation was implicated in vertebral fusion of Atlantic salmon [36,37]. We previously reported that the 2-kb *Matn1* promoter region directs transgene expression to the GP zones that specifically express endogenous *Matn1* [20,21]. This promoter region features several sequence blocks that are highly conserved in amniotes [38], and we showed that the most proximal elements, *i.e.*, a highly conserved promoter element 1 (Pe1), an initiator element (Ine), and two silencers (SI and SII), function together to restrict promoter activity in a stage- and GP zone-specific manner [3,38,39]. This is made possible by a dose-dependent modulation of the activity of Sox9, bound the Pe1, by L-Sox5/Sox6, bound to Ine, and nuclear factor I (Nfi) family proteins, bound to SI and SII.

Here we extend our model demonstrating that a distal promoter element 1 (Dpe1), which functions as an enhancer in transgenic mice, interacts with the short promoter *via* the Sox trio. Furthermore, we show that Hmgb1, whose expression overlaps with that of *Sox9* in early chondrogenesis, is capable of increasing the activation of the *Matn1* promoter by the Sox trio, whereas it represses *Matn1* expression in later steps or in established cell lines.

2. Materials and methods

2.1. Cell culture

Chicken embryo fibroblasts (CEF), chondroblasts (CEC), and mesenchymal cells were prepared and cultured as described previously [39]. Low density (LDM) and high-density mesenchyme (HDM) cultures were made by plating 1×10^6 cells and 5×10^6 cells, respectively, in 35-mm plates as described previously [3]. COS-7 cells were cultured under standard conditions. HDM cultures consisting of early proliferative (stage Ia) chondroblasts and CEC cultures rich in late proliferative (stage Ib) chondroblasts represented the low and high *Matn1*-expressing cell types, respectively [17,18,39]. LDM, CEF, COS-7, and human 293T cultures were used as *Matn1*-nonexpressing controls.

The C-28/I2 immortalized human costal chondrocyte [40], the SW1353 human chondrosarcoma (ATCC HTB-94) and the RCS (rat chondrosarcoma) [41] cell lines were cultured in DMEM supplemented with 10% FCS (GIBCO).

2.2. Oligonucleotides and plasmid constructions

As previously, all positions are given in bp from the first T of the TATA motif of the chicken *Matn1*. Luciferase reporters *FO15Luc* and *AC8Luc* driven by the short and long *Matn1* promoters, respectively, were described [38] as well as their mutant derivatives Δ*Pe1M1*-, Δ*Pe1M4*-, Δ*IneM1*-, Δ*IneM2*-, Δ*IneM3*- and Δ*Pe1M1*-Δ*IneM2*-*AC8Luc* [3]. ΔDpe1ABC-, ΔDpe1BC- and ΔDpe2-AC8Luc were made by deleting sequences between positions −1879/−1791, −1848/−1791 and −1745/−1642, respectively, from the long *Matn1* promoter.

Luciferase reporters harboring multiple copies of the Dpe1 element upstream of the *Matn1* short promoter were made by inserting one to eight copies of the PCR-amplified Dpe1 fragment into *FO15Luc*. *4×Dpe1(−)FO15Luc* was generated by inserting blunted four copies of Dpe1 into *FO15Luc* in reverse orientation. *PCLuc* and *4×Dpe1(+)PCLuc* were generated by replacing the *Matn1* short promoter of *FO15Luc* and *4×Dpe1(+)FO15Luc*, respectively, with the *Col2a1* short promoter fragment between positions −309/+118.

For transgenic experiments, *LacZ* fusion construct 8×Dpe1(+) NAD1 was produced by inserting eight copies of the Dpe1 element upstream of the short promoter (−334/+67) of NAD1, the *Matn1* short promoter-*LacZ* reporter [38].

Structures and sequences of all constructs were verified by restriction mapping and sequencing.

2.3. Transient expression assays

CEC and CEF cultures were transfected with 2 μg reporters, while HDM, LDM, and COS-7 cultures were transfected with 5 μg reporters