Role of Sox trio, Nfi and Hmgb1 factors in the unique transcriptional regulatory mechanisms of the matrilin-1 gene

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PhD Thesis

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1. LIST OF PUBLICATIONS

Articles related to the subject of the Thesis

I. Nagy A, Kénesi E, Rentsendorj O, Molnár A, **Szénási T**, Sinko I, Zvara Á, Oommen ST, Barta E, Puskás LG, Lefebvre V, Kiss I
Evolutionarily conserved, growth plate zone-specific regulation of the matrilin-1 promoter: L-Sox5/Sox6 and Nfi factors bound near TATA finely tune activation by Sox9.
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II. **Szénási T**, Kénesi E, Nagy A, Molnár A, Bálint BL, Zvara A, Csabai Z, Deák F, Boros Oláh B, Mátés L, Nagy L, Puskás LG, Kiss I
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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ABBREVIATIONS

Acan	Aggrecan gene
BMP	bone morphogenetic protein
CD-RAP	cartilage-derived retinoic acid-sensitive protein
CEC	chicken embryo chondrocytes
CEF	chicken embryo fibroblasts
Col2a1	collagen-2 gene
DMEM	Dulbecco's modified Eagle's medium
Dpe1 and Dpe2	distal elements 1 and 2
DTT	dithiothreitol
ds	double stranded
ECM	extracellular matrix
EMSA	electrophoretic mobility shift assay
FCS	fetal calf serum
GST	glutathione S-transferase
GTF	general transcription factor
HDM	high-density mesenhyme
HMG	high-mobility group
Ine	initiator element
IPTG	isopropyl-β-D-thiogalactopyranoside
MATN1/Matn1	matrilin-1 (human/other vertebrate)
Matn2	matrilin-2 (human/other vertebrate)
MATN3/Matn3	matrilin-3 (human/other vertebrate)
MED	multiple epiphyseal dysplasia
NFI/Nfi	nuclear factor-I (human/other vertebrate)
PAGE	polyacrylamid gel electrophoresis
Pe1	promoter element 1
PIC	preinitiation complex
PMSF	phenylmethylsulphonylfluoride
SF-1	steroidogenic factor-1
SI and SII	silencer element I and II
Sox	sry-type HMG box protein
Sry	sex-determining region Y
TBP	TATA-binding protein

2. INTRODUCTION

2.1. Cartilage and chondrogenesis

2.1.1. Endochondral bone formation

Endochondral ossification is a multistep process, which involves spatiotemporally strictly regulated morphogenetic and differentiation steps [1, 2]. It leads to the formation of most of the skeletal elements during vertebrate embryonic development and it takes place also in the growth plates, where it is responsible for longitudinal bone growth till adulthood. During endochondral ossification mesenchymal precursors differentiate into bone tissue through replacement of a cartilaginous intermediate.

Chondrogenesis starts with the condensation and commitment of mesenchymal precursors and their differentiation to prechondrocytes in a shape prefiguring the future bones (Fig 1). The prechondrocytes subsequently differentiate into early chondroblasts, which proliferate, form columns and deposit an extensive cartilaginous extracellular matrix (ECM) serving as template of the future bones. Then, starting from the middle of the cartilaginous primordia, proliferative chondroblasts exit from the cell cycle and successively undergo prehypertrophic and hypertrophic development [3, 4]. Finally blood vessels, osteoclasts and osteoblasts invade and replace the mineralized hypertrophic cartilage by bone tissue. Growth plates are formed at the end of long bones by chondrocytes organized into distinct zones according to their differentiation stages. During fetal and postnatal development, bones elongate as the cells proliferating in the columnar zone progress through the prehypertrophic and hypertrophic zones, and eventually the calcified ECM laid down by terminally differentiated cells is used as a scaffold by osteoblasts to deposit bone ECM.

Chondrogenesis is directed by three master transcription factors, Sox9, L-Sox5 and Sox6, called the Sox trio (Fig 1) (see later 2.2.3.) [5]. Numerous autocrine, paracrine and endocrine factors determine the shape and size of skeletal elements often acting by modulating the expression or the activity of the Sox trio. Maintenance of proliferation in the columnar zone and bone growth depends on positive and negative feed back loop formed between Ihh (Indian hedgehog) and PTHrP (parathyroid hormone-related protein) and affected by growth factors (FGF, BMP), hormones and various other signaling pathways [2, 3, 6]. Stimulation of chondrocyte proliferation by mechanical load is mediated by Ihh via BMP signaling [7]. On the other hand, induction of Ihh expression in the prehypertrophic zone, hypertrophic differentiation and ossification is regulated by Runx2, the master factor of the osteoblast lineage [6]. In addition,

Hmgb1 secreted by hypertrophic growth plate chondrocytes is needed for cell invasion to function as a chemoattractant for osteoclasts, osteoblasts and endothelial cells [8].

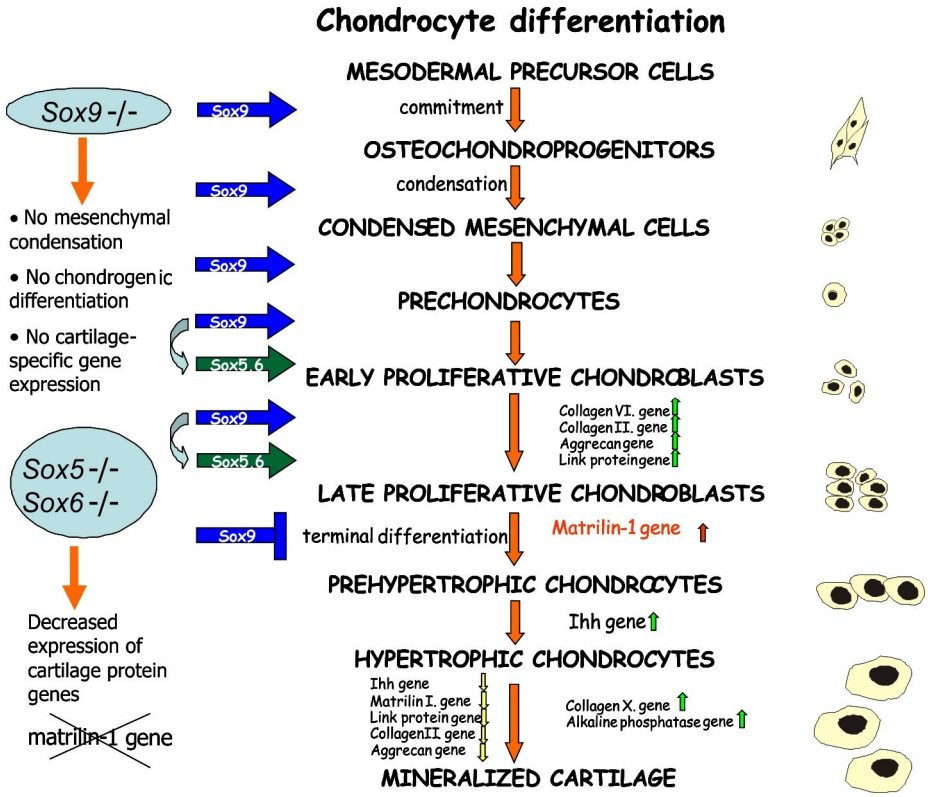

Fig. 1. Chondrogenesis. Stages of chondrocyte differentiation are shown indicating the up- and down-regulation of important marker genes by green and yellow arrows, respectively. Role of Sox transcription factors in the regulation of differentiation steps is also indicated.

2.1.2. Gene expression during chondrogenesis

The **cartilaginous ECM** consists mainly of proteoglycans, collagens and non-collagenous multiadhesion proteins. While the major constituent, collagens and the proteoglycan-hyaluronan aggregate determine the physical properties of the cartilaginous ECM, other components are responsible for the diversity among growth plate zones and cartilage forms. The collagen fiber network comprised of collagen-2, collagen-9 and collagen-11 is responsible for the high tensile strength of cartilage. The collagen network in cartilage entraps a highly hydrated gel of proteoglycans and glycoproteins giving high resilience to cartilage against compression and shear forces. Aggrecan (Acan) is the most abundant proteoglycan that is almost unique to cartilage. Acan molecules form enormous aggregates by binding to linear chains of the glycosaminoglycan hyaluronan with the help of cartilage link protein (Crtl1). Glycoproteins, such as matrilins, cartilage oligomeric matrix protein (Comp) and small proteoglycans, such as fibromodulin,

decorin and biglycan, vary in abundance according to the types of cartilage. Collagen-10 is produced in abundance exclusively by prehypertrophic and hypertrophic chondrocytes.

Matrilins are multidomain filament-forming proteins, which function as adaptor proteins in the ECM assembly and in mechanotransduction of chondrocytes [9-11]. **Matrilin-1 (Matn1)** was formerly called CMP (cartilage matrix protein) as it is expressed exclusively in chondrocytes, while Matn2, Matn3 and Matn4 are also found in other tissues. By binding many ECM components, Matn1 can mediate connections between Acan, collagen-2 fibers and collagen-6 beaded filaments. Lack of functional Matn1 and Matn3 in the pericellular matrix was reported to interfere with Ihh signaling and mechanical stimulation of chondrocyte proliferation and differentiation [12].

Chondrogenesis is accompanied by **sequential changes in the ECM gene expression** and in the ECM composition [3, 13, 14]. Thus prechondrocytes start to express *Col2a1* (collagen-2 gene), whereas genes for aggrecan (*Acan*), minor collagens (e.g. *Col9a1*) and non-collagenous proteins (e.g. *Crtl1*) are turned on in early chondroblasts and activated further in columnar chondroblasts. *Matn1*, however, has a narrower expression pattern in both space and time than other ECM genes [14-17]. In particular, its expression is restricted to the columnar and prehypertrophic growth plate zones playing important roles in bone growth [18-20]. In chondrogenic cultures, it showed a delayed activation in early proliferative chondroblasts as compared to other cartilage ECM genes and had a high activity only in late proliferative chondroblasts [16]. During terminal differentiation, the cartilage-specific genes are turned off, while *Col10a1* (collagen-10 gene) is turned on.

2.1.3. Effect of ECM gene mutations in animal models of human diseases

Lack or mutations of genes encoding the major ECM components, which determine the main physical properties of cartilage, such as collagen-2, Acan and Crtl1, cause serious or lethal diseases. Thus, null mutation for *COL2A1*/*Col2a1* leads to lethality or severe abnormalities in both human and mouse skeletal development [21]. This disease is called achondrogenesis type II, which is characterized by short limbs, ribs and trunk and domed skull in collagen-2-null mice [22]. In addition, glycine mutatitions in collagen-2 generate a range of chondrodysplasias, including spondyloepiphyseal dysplasia, Kniest dysplasia, hypochondrogeneis and Stickler syndrome (premature osteoarthritis) [22, 23]. A 7-bp deletion in *Acan* leading to stop codon in exon 6 caused cartilage matrix deficiency (cmd) in the mouse [21], in the animal model of lethal human chondrodysplasia. Homozygous mice for cmd die at birth and suffer from disproportionate dwarfism, clef palate, short snout and protruding tongue. Targeted inactivation

of *Crtl1* in transgenic mice revealed the essential role of the protein in cartilage development. Most homozygotes died shortly after birth due to respiratory failure and the few survivors developed progressive drawfism, craniofacial abnormalities and lordosis of the cervical spine. They showed characteristics of spondyloepiphyseal dysplasias, such as small epiphysis and flattened vertebrae [24].

Mutations in other cartilage proteins involved in the ECM assembly or disruption of their genes have less dramatic effects in animal models. For instance, mice deficient in matrilin family members are viable under laboratory conditions [10]. *Matn1* upregulation was implicated in vertebral fusion of Atlantic salmon [25]. A linkage of *MATN1* was reported to osteoarthritis in the Dutch population [26]. Various polymorphism of *MATN1* was found to predispose to idiopathic scoliosis in Asian and Turkish population [27]. *MATN1* polymorphism was also linked to mandibular prognatism in human [28]. *MATN1* was clearly associated to relapsing polychondritis, a rare autoimmune disease. Circulating MATN1 antibodies were found to function as autoantigen in these patients, and immunization of mouse or rat with MATN1 caused similar symptoms in an animal model of this human disease [29, 30].

Among matrilins, a close association with human diseases has been best characterized for MATN3. Mutations in MATN3 caused a severe disease referred to as pseudoachondroplasia (PSACH) or a milder form called multiple epiphyseal dysplasia (MED). These are characterized by moderate disproportionate dwarfism and musculoskeletal alterations. In the milder forms of MED mutations were identified in *MATN3* or collagen-9 genes (*COL9A1*, *COL9A2*, *COL9A3*), whereas the severe form was caused by mutations in *COMP* (cartilage oligomeric matrix protein gene) [31]. More than 20 different mutations within *Matn3* were connected to autosomal-dominant MED. A mouse model of MED caused by a *Matn3* V194D mutation replicated the human phenotype [32]. Homozygous mice for this mutation are normal at birth but from day 14 they develop a measurable short-limbed dwarfism as a result of disturbed endochondral ossification. Interestingly, when mice with a Matn3 V194D mutation were crossed with Matn1-null mice, aggravation of the skeletal phenotype was observed [33].

Matn2$^{-/-}$ mice do not show obvious skeletal abnormalities [34], but they serve as a good animal model of DEN-induced hepatocarcinogenesis [35, 36]. This animal model is suitable to assess the efficacy of antitumor drugs [37]. Matn2 also plays a role in myogenesis timing during muscle regeneration [38].

2.2. Transcriptional control of tissue-specific gene expression

2.2.1. Players of eukaryotic transcription

In eukaryotes, the template for mRNA transcription is not free DNA, but chromatin. This is the highest level of eukaryotic gene regulation, and chromatin has a major regulatory role in development. The chromatin contains histone and non-histone components. The core **histones** (H2A, H2B, H3 and H4) package the DNA to make up the basic chromatin unit, the nucleosomes, which are linked by histone H1, the linker histone and assembled into higher-order structure Gene activation requires various chromatin remodeling processes to open the compacted chromatin into a relatively extended state and to expose specific DNA sequences so that these can be targeted by the RNA polymerase II (PolII) machinery and transcription factors involved in the process of gene transcription.

The **non-histone chromatin components** function as architectural factors in the organization and fluidization of the chromatin by appropriately bending and plasticizing DNA. For instance, the high mobility group (**HMG**) **proteins** belong to three families based on their DNA-binding domains. Hmga proteins interact with AT-rich sequences in the minor groove of the DNA by their AT-hook HMGA domain. Hmgb proteins bind DNA without sequence specifity in the minor groove by their L-shaped HMG box (HMGB) domains. Hmgn proteins directly interact with nucleosomes via their nucleosome-like HMGN domain. HMGBs better bend the DNA than transcription factors that bind DNA in the major groove.

Transcriptional control represents the second, best characterized level in the spatio-temporal regulation of gene expression. Transcription occurs as a result of interplay between *cis*-acting DNA elements and *trans*-acting factors. The ***cis* elements** include the core promoter, promoter-proximal and distal control elements (e.g. enhancers and silencers), which carry binding sites for various ***trans*-acting factors**. Apart from PolII, *trans*-acting factors include general transcription factors (GTFs) (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH), associated factors (TAFs), the mediator complex and classical transcription factors. A typical core promoter features a TATA box 25-30 bp upstream of the transcription start site. The TATA box is recognized by the TATA-binding protein (TBP) of the TFIID complex, whereas TAFs and PolII interact with the initiator element.

The general transcription machinery consisting of PolII, GTFs and the mediator complex can assemble on the core promoter and form a preinitiation complex (PIC), but it can drive only a basal level of transcription. **Classical transcription factors** (e.g. NFI, IL1, HOX and POU proteins) recognizing proximal and distal control elements are required for activated or regulated

transcription to increase the efficiency of initiation. They bind short DNA motifs in the major groove of DNA with high sequence specificity and high affinity. Based on their DNA binding domains, we distinguish zinc finger, helix-loop-helix, helix-turn-helix, leucine zipper and homeodomain proteins. The classical or typical transcription factors also feature activation or repression domains and by interacting with GTFs (e.g. TFIIA) they can either serve as activators by activating transcription, or as repressors by inhibiting transcription [39]. The classical transcription factors often have dimerization domain and they may function as cell type-specific or ubiquitous factors. They often bind in a cooperative manner to nearby DNA elements.

According to latest scientific results, there are *trans*-acting factors, which represent a transition between architectural HMG proteins and classical transcription factors. For instance the Sry-related high mobility group box (**Sox**) **proteins** play critical roles in cell fate and differentiation decisions in various lineages [3, 40, 41]. Whereas Hmgb proteins are the most abundant non-histone chromatin components, Sox proteins are expressed at a low level and only in certain cell types. They bind DNA via their HMGB domain in the minor groove with low affinity and sequence specificity, but with lower sequence specificity and lower binding efficiency (K_d~10^{-9}-10^{-11}) than classical transcription factors (K_d~10^{-7}-10^{-9}). Like Hmgb proteins, Sox factors also bend the DNA as architectural proteins, thereby facilitating the binding of other factors. Some of them have activation or repression domain and can directly influence transcription like classical transcription factors.

Proximal and distal *cis*-control elements are critical players of tissue-specific gene regulation. The proximal DNA elements can function in one orientation and only within a few hundred bp from the TATA box. They usually have binding sites for some ubiquitous and tissue-specific factors. By contrast, **enhancers** and **silencers** highly activate or repress transcription, respectively, over large distances and independent of orientation. They usually carry an array of recognition motifs for a unique set of transcription factors, which bind in a cooperative manner and interact with each other and other factors and GTFs bound to proximal DNA elements and the core promoter. Thus, enhancers perform a crucial function in the formation of stereospecific nucleoprotein complexes, the enhanceosomes, which modulate transcription via very complex protein-protein and protein-DNA interactions. Tissue-specific gene regulation is based on a combinatorial interaction between *cis*-acting elements and *trans*-acting factors. Apart from classical transcription factors, recent data underline the important role of SOX proteins and canonical HMGB proteins in the regulation of gene expression during development and disease.

2.2.2. Role of Hmgb1 in gene regulation, development and disease

Hmgb proteins are very abundant chromatin components. They consist of an acidic tail and two L-shaped archetypal HMG-boxes, which bind distorted DNA (bent, kinked, four-way junctions) without sequence specificity and only transiently [42-45]. Lacking transactivation domain, they act only as architectural proteins to alter the chromatin structure and modulate transcription. Hmgb1, which is only 10 times less abundant than core histones, can promote the transcription of genes by several mechanisms. It interacts with nucleosomes via its acidic tail and loosens the nucleosome structure by displacing histone H1 due to competition for closely juxtaposed binding sites on linker DNA [44, 46]. Thus fluidizing the chromatin, Hmgb1 can increase the accessibility of chromosomal DNA to regulatory factors. By bending the promoter, it helps recruit TBP and other GTFs [43]. By bending the DNA and making protein-protein interactions, Hmgb1 can enhance and stabilize the binding of various transcription factors (e.g. steroid receptor, Hox and Pou proteins) to their cognate sites and promote the recruitment of additional interacting factors.

Hmgb1 performs diverse architectural and extracellular function in health and disease. In the nucleus, it regulates chromatin architecture, transcription, replication and repair processes. In addition, Hmgb1 secreted by macrophages and necrotic cells also performs various intracellular and extracellular activities as an alarmin, a danger signal during injury and diseases, such as infection, inflammation, arthritis and cancer [47]. Hmgb1 was also implicated recently in skeletal morphogenesis [48] and in the regulation of late steps of endochondral ossification [8], but its role in early steps has not been investigated yet.

2.2.3. Role of Sox proteins in cell fate determination and tissue-specific gene regulation

Sox proteins play critical roles in cell fate and differentiation decisions in various lineages [3, 40, 41]. Differing from Hmgb proteins, Sox proteins have low abundance and contain a single, Sry-related, non-canonical HMG-box, which binds DNA in the minor groove with low affinity and loose sequence specificity [49]. They recognize the A/TA/TCAAA/T motif. Sox factors likely bend the DNA as architectural proteins to facilitate the binding of other factors. Some (e.g. Sox9) can directly influence transcription via their activation/repression domain and pair off with various transcription factors. They interact with many partner factors to facilitate enhanceosome formation and control cell fate and differentiation during vertebrate development [50]. Sox proteins are encoded by 20 genes and classified into 8 groups based on the similarity of their HMGB domains. Sox factors play crucial role in the development of various organs during

embryogenesis. For example Sry and Sox9 function in sex determination, Sox1, Sox2 and Sox3 are involved in eye development and neurogenesis, whereas Sox4, Sox11 and Sox7 have been implicated in cardiogenesis [50]. Three Sox proteins (Sox9, L-Sox5 and Sox6), the so called **Sox trio** play a well established role in endochondral bone formation by defining the chondrocyte lineage, regulating chondrogenesis and turning on cartilage-specific genes (Fig. 1) [50] **Sox9** serve as a master regulator of the chondrocyte lineage [51]. It is expressed from mesodermal precusor cells to the prehypertrophic chondrocyte stage. It directs the commitment of osteochondroprogenitors and their differentiation to prechondrocytes. Sox9 is required for the aggregation of mesodermal precursor cells and it turns on the genes for L-Sox5 and Sox6 and they together direct subsequent steps of chondrogenesis (Fig. 1). Sox9 is also required for the activation of cartilage protein genes such as *Col2a1, Col9a1*, *Col11a2* and *Acan* [52, 53].

The first clue that Sox9 plays a role in chondrogenesis came with the identification of heterozygous mutations in the *SOX9* gene in human patients with camptomelic dysplasia (CD), a severe form of chondrodysplasia that is often associated with XY sex reversal and malformations in several internal organs [54, 55]. In addittion, Sox9 pairs off with steroidogenic factor 1 to regulate transcription of the anti-Muellerian hormone (AMH) gene involved in sex determination [56].

L-Sox5 and **Sox6** are expressed in early proliferative chondroblasts and they are required for the differentiation of early chondroblasts to late proliferative or columnar chondroblasts in the growth plate. L-Sox5 and Sox6 are also needed for the activation of some of the cartilage ECM genes (*Comp*, *Matn1*) and for the high activity of others (*Col2al*, *Acan*, etc.) L-Sox5 is a longer product of the *Sox5* gene. It is chondrocyte-specific splice variant and highly similar to Sox6. Sox5-null and Sox6-null mice are born with minor cartilage defects, whereas the double-null mice develop a severe, generalized chondrodysplasia and die around embryonic day 16.5 [57]. Respectively, these mice have impairment of chondroblast proliferation and expression of cartilage matrix genes. Interestingly, L-Sox5 and Sox6 are required to turn on *Matn1*, as *Matn1* mRNA was not detected in $Sox5^{-/-}$; $Sox6^{-/-}$ mice (Fig. 1) [57].

2.2.4. Role of Nfi proteins in development and tissue-specific gene regulation

Nuclear factor I (NFI/Nfi) is a family of closely related transcription factors, also known as CTF or CAAT box transcription factors. CTF/NFI is constitutively active classical transcription factor which binds to the CCAAT box motif in the DNA major groove [39]. NFI family members play diverse roles in adenovirus DNA replication [58] and transcriptional regulation of viral and eukaryotic genes as activators or repressor [59]. They are composed of N-terminal DNA-

binding/dimerization domain and C-terminal, proline-rich transcriptional activation or repression domain [60, 61]. They bind to the consensus sequence TTGGC(N5-7)GCCAA) as both homodrimers and heterodimers [59, 62]. In vertebrates, the protein family is composed of four members Nfia, Nfib, Nfic and Nfix encoded by four different genes located on three chromosomes in mammals. The expression patterns of the four Nfi genes overlap during mouse embryogenesis [63]. Nfi proteins play relevant role in brain, lung, tooth and skeletal muscle development [64-68]. In addition, Nfib has been implicated in early stage of chondrogenesis and in the regulation of *Col2a1* transcription [69]. Further, NFI proteins can activate transcription through direct interaction with basal transcription factors (TFIIB and TBP) [70, 71], various coactivator or corepressor factors [72] and histone proteins (H1 and H3) [60, 73] via their proline-rich transactivation domain.

2.3. Regulation of cartilage-specific gene expression

2.3.1. Role of the Sox trio in the transcriptional regulation of cartilage ECM genes

The Sox trio plays essential role in the transcriptional regulation of cartilage protein genes. Sox9, L-Sox5 and Sox6, besides specifying other lineages, are required and sufficient to induce chondrogenesis and cartilage gene expression [50, 53, 57, 74]. Sox9 binds and activates cartilage-specific enhancers in *Col2a1* [75], *Col11a2* [76] and *CD-RAP* [77]. Besides a DNA-binding HMGB domain, Sox9 also contains a transactivation domain and a DNA-dependent dimerization domain similarly to other members of Group E Sox proteins. Group D Sox proteins, L-Sox5 and Sox6 have no transactivation domain and coiled-coil domain mediates their homo- and heterodimerization [49].

The 48-bp intronic enhancer element was identified in the first intron of *Col2a1* [75], which contains three Sox binding site [52]. These elements bind SOX9, L-Sox5 and Sox6 in a cooperative manner [52]. Multiple copies of this element enhanced homologous and heterologous promoter activity in the developing cartilaginous skeletal elements of transgenic mouse embryos [78]. This DNA element is sufficient to drive cartilage-specific gene expression [75]. The *COL9A1* enhancer contains two tandem pairs of consensus Sox motifs. SOX9 binds to the enhancer and two interdependent dimers are required for transcriptional activation [79, 80]. The new collagen gene *COL27A1* also carries SOX9-responsive enhancer elements involved in the transcriptional regulation of the gene [81]. Sox consensus binding sites activated by SOX9 were also found in the cartilage-derived retinoic acid-sensitive protein gene (*CD-RAP*) [77]. The

enhancer and other cis elements from the *Col11a2*, *Col27A*, *Col2a1*, *Col9a2* and *CD-RAP* contain two Sox motifs arranged in opposite orientation to each other and separated by 3 or 4 bp [77, 78, 81-83]. Sox9 binds as a homodimer, but L-Sox5 and Sox6 bind as a heterodimer to these binding sites [49, 83]. In contrast, the non-cartilage-specific enhancers from the Steroidogenic Factor I (*SFI*) and the anti-Mullerian hormone genes (*AMH*) contain one SOX9 binding site and they are activated by SOX9 as a monomer and only [83]. Thus, dimerization of SOX9 is required for chondrogenesis, but not for sex determination and gonad development [83].

L-Sox5/Sox6 synergize with Sox9 in the transcriptional control of *Col2a1* and *Acan* [52, 84]. However, it is far from being understood, how the same Sox trio can exert distinctive regulation to other cartilage protein genes with markedly different spatio-temporal expression pattern during skeletogenesis.

2.3.2. Transcriptional regulation of *Matn1*

Previously, our group cloned the gene for the chicken Matn1 [85]. This gene is 18 kb long and comprises 8 exons and 7 introns [85]. The chicken *Matn1* promoter contains a TATA motif, TTAATA, which can function as a minimal promoter in transient expression assay [85]. The *Matn1* promoter exhibited chondroblast stage-specific activity in transient transfections [86, 87].

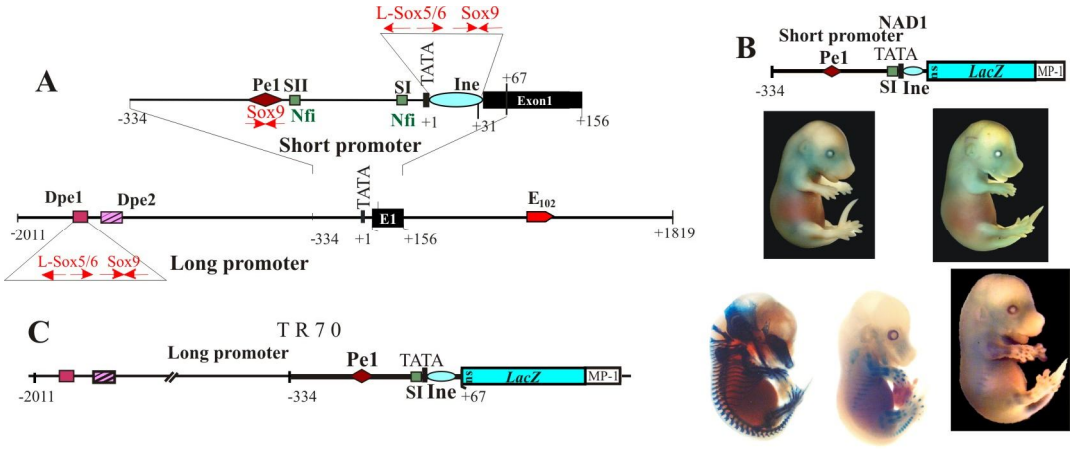

Fig. 2. Map of conserved control elements of the chicken *Matn1* and their functional analysis in transgenic mice. (A) The schematic of the *Matn1* promoter region illustrates the intiator element (Ine), the silencer elements (SI and SII), the proximal promoter element (Pe1) and distal promoter upstream elements (Dpe1 and Dpe2). Transcription factors binding to the conserved elements are represented by factor names. (B and C) Map of the constructs and functional analysis of the short (B) and long promoter (C) in transgenic mice.

The chicken *Matn1* exhibited a characteristic zonal expression pattern in the developing skeleton of transgenic mice [20]. The transgene expression was restricted to the zones of

columnar proliferating chondroblasts and prehypertrophic chondrocytes of growth plate cartilage. The 2-kb *Matn1* promoter with or without the intronic enhancer also directed zonal transgene expression. Resembling to the expression pattern of the endogenous *Matn1*, LacZ expression was highest in the columnar and prehypertrophic growth plate zones and it exhibited proximo-distal differences in the developing skeleton of transgenic mice (Fig 2C) [20]. The 334-bp short promoter with the intronic enhancer showed low activity, but of similar pattern, whereas the short promoter alone directed hardly detectable transgene activity [20, 86].

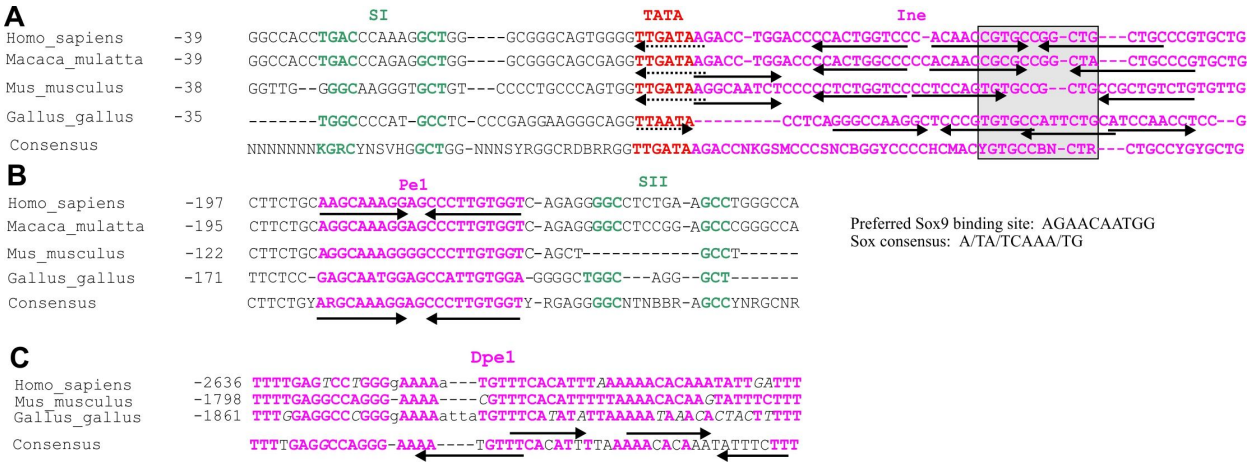

Fig. 3. Conservation of DNA elements in the *Matn1* promoter in amniotes. Sequence alignments of the promoter in the vicinity of the TATA box and Ine (A), the Pe1 (B) and Dpe1 elements (C) are shown from a few mammalian species and the chicken. Numbers indicate positions from TATA. Arrows and dotted arrows indicate inverted Sox motifs similar to the preferred Sox9-binding site and the Sox consensus, respectively. The most conserved motif of Ine is boxed. Conserved motifs similar to the Nfi consensus sequence TTGGC(N5-7)GCCAA appear in green.

The long (2-kb) *Matn1* promoter region features several sequence blocks which are highly conserved in amniotes (Fig2A and 3) [19, 85, 86]. These include the most highly conserved proximal promoter element 1 (Pe1), which was previously identified by our group in the short promoter. It was reported that Pe1 is recognized by the Sox trio, whereas the silencer elements SI and SII bind Nfi proteins [87] (Fig. 2B). Furthermore, the 2-kb long *Matn1* promoter also includes two conserved distal promoter elements, distal promoter element 1 (Dpe1) and distal promoter element 2 (Dpe2) (Fig.2A). In transient expression assay, multiple copies of the Dpe1 element robustly increased the short promoter activity in CEC culture [88]. Eight copies of Dpe1 fused to the short promoter directed zonal transgene expression of high level in distal structures of the developing skeletal elements in transgenic mice [88].

3. AIMS OF THE STUDY

Among the cartilage protein genes, *Matn1* has a unique expression pattern, restricted to certain chondrocyte developmental stages and distinct zones of the growth plate. This suggests that regulation of the *Matn1* may involve similar as well as different molecular mechanism as compared to other cartilage protein genes, e.g. *Col2a1*. To get insight into the common and distinct molecular mechanisms controlling cartilage-specific gene expression, we aimed to identify DNA elements and uncover the role of transcription factors in the transcriptional regulation mechanism of *Matn1*.

The following specific goals were set out:

1. We planned to study the contribution of the Sox trio, Nfi and Hmgb1 proteins to the transcriptional activity of the *Matn1* promoter.
2. We planned to verify that the conserved Dpe1 element is operable as an enhancer.
3. We planned to study the role of Hmgb1 in the early steps of chondrogenesis.
4. We planned to delineate a hypothesized model of *Matn1* transcriptional regulation.

4. MATERIALS AND METHODS

4.1. Cell culture

Chicken embryo chondroblast (CEC), fibroblast (CEF) and high-density mesenchyme (HDM) cultures undergoing chondrogenesis in vitro were prepared as described [86, 89]. CEC cultures were obtained from sterna of day 14.5 embryos using 0.1 % collagenase treatment. CEF cultures were prepared from 8-10 day embryos by trypsin treatment. HDM cultures were made from the limb buds of stage 23-24 chicken embryos. Low-density mesenchyme (LDM) cultures were made similarly as HDM cultures, but instead of $5x10^6$ cells, only $1x10^6$ cells were plated in 35-mm plates in F12/DMEM 1:1 (HyClone Laboratories) supplemented with 10% FBS (Sigma and GIBCO Laboratories). 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 [16, 17, 87]. LDM, CEF, COS-7 cultures were used as *Matn1*-nonexpressing controls. The C-28I/2 immortalized human costal chondrocyte [90], the SW1353 human chondrosarcoma (ATCC HTB-94) and the RCS (rat chondrosarcoma) [91] cell lines were cultured in DMEM supplemented with 10% FCS (GIBCO).

4.2. Quantitative real-time PCR (QRT-PCR)

Total RNA was isolated from CEC, CEF or HDM cultures at subsequent days of chondrogenesis using the RNA isolation kit (Macherey-Nagel) according to the manufacturer's instructions. The quantity of isolated RNA was measured by spectrophotometry (NanoDrop). QRT-PCR was performed on a RotorGene 3000 instrument (Corbett Research) with gene-specific primers (Suppl. Table S1) and SybrGreen protocol to monitor gene expression changes. The primers were designed by the Roche online primers design tool (https://www.roche-applied-science.com/sis/rtpcr/upl). Briefly, 2 μg of DNase-treated total RNA from each sample was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. Reactions were done with FastStart SYBR Green Master mix (Roche Applied Science) at a final primer concentration of 250 nM as follows: 15 min at 95 ^oC, 45 cycles of 95 ^oC for 15 s, 60 ^oC for 25 s and 72 ^oC for 25 s. The quality of the reaction was checked by melting temperature analysis after each reaction. The quality of primers was verified by MS analysis provided by Bioneer (Daejeon). Each individual $C\tau$ values were normalized to the average $C\tau$ values of three internal control genes (GAPDH, 18S rRNA, and 28S rRNA). The final relative gene expression ratios were calculated as either $2^{-\Delta C\tau}$ values (compared to the

internal control genes) or $2^{-\Delta\Delta C\tau}$ values (comparison of the normalized ratios) as indicated in the figure legend.

4.3. Plasmid constructions

All positions are given in bp from the first T of the chicken *Matn1* TATA motif. Luciferase reporters *FO15Luc* and *AC8Luc* driven by the short and long *Matn1* promoters, respectively, as well as Δ*Pe1M1- AC8Luc* and Δ*Pe1M4- AC85Luc* carrying point mutations in the Sox motif and spacer of Pe1, respectively, were described [86, 89]. To produce *8x*E_{Col2a1}*-FO15Luc*, eight copies of E_{Col2a1} were ligated to the SmaI site of *FO15Luc*. *AC8Luc* derivatives Δ*IneM1-,* Δ*IneM2-*, Δ*IneM3-* and Δ*SI2dm-AC8Luc* were made by the PCR-based QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions using *AC8Luc* as template and oligonucleotides IneM1, IneM2, IneM3 and P1-2d carrying mutation in the Nfi-binding site of SI [87-89], respectively. Double mutants Δ*Pe1M1*-Δ*IneM2-*, Δ*Pe1M1*-Δ*SI2dm-* and Δ*Pe1M4*-Δ*SI2dm- AC8Luc* were constructed similarly using Δ*Pe1M1-* and Δ*Pe1M4-FO15Luc* DNAs as templates. ΔDpe1ABC- and ΔDpe1BC- were made by deleting sequences between positions −1879/−1791 and −1848/−1791, respectively, from the long *Matn1* promoter. Mutant derivatives of *8x*E_{Col2a1}*-FO15Luc* were made by replacing the wild-type short promoter of *8x*E_{Col2a1}*-FO15Luc* with the corresponding mutant *FO15Luc* fragment. Structures and sequences of all constructs were verified. Luciferase reporters harboring multiple copies of the Dpe1 element upstream of the *Matn1* short promoter were made by inserting four copies of the PCR-amplified Dpe1 fragment into *FO15Luc* [88]. *4×Dpe1(−)FO15Luc* was generated by inserting blunted four copies of Dpe1 into *FO15Luc* in reverse orientation [92]. *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. Structures and sequences of all constructs were verified by restriction mapping and sequencing.

4.4. 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 using the Ca-phosphate coprecipitation method as described previously [86, 88, 89]. Cotransfection with pRL-TK (Promega) served as an internal control to correct for transfection efficiency, but parallel plates were also transfected with *FO15Luc*. Firefly and renilla luciferase activities were measured in a Luminoscan Ascent

(ThermoLabsystem 2.6) using the respective Luciferase Assay Systems (Promega) according to the supplier's instructions 72 h (HDM and LDM) or 48 h (other cells) posttransfection. Relative luciferase activities were expressed in fold as compared to values of *FO15Luc* taken as 1, unless noted otherwise.

Cotransfection assays were performed with 2 µg or 5 µg *AC8Luc* reporter using effector plasmids pcDNA5'UT-FLAG-L-Sox5 (pFSox5), pcDNA5'UT-FLAG-Sox6 (pFSox6), and pCDMA-SOX9 (pSOX9) or pcDNA5'UT-FLAG-SOX9 (pFSOX9) [52], plasmids expressing Nfi factors and pHmgb1 expressing rat Hmgb1 [93]. In a typical experiment, 125 ng pFSox5, 125 ng pFSox6 and 250 ng pSOX9 or 250 ng pFSOX9 were added with or without 100 ng pHmgb1. Some experiments were performed with increasing amounts of pFSox5 and pFSox6 (0-250 ng) or pHmgb1 (0-500 ng). Other experiments were performed with 0 to 300 ng effector plasmids expressing mouse Nfia, Nfib, Nfic, and Nfix (pNfia, pNfib, pNfic and pNfix), homologous to chicken Nfia1.1, Nfib2, Nfic2, and human NFIX2, respectively [63]. All transfections were done in duplicates or triplicates and repeated 3-10 times with at least two different DNA preparations. Results are presented as means ± standard error of the mean (SEM).

Statistical analysis was carried out using one-way analysis of variance (ANOVA) and Dunett's test with KyPlot version 2.0 beta 15. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. reporters cotransfected with empty vector(s) or mutants vs. similarly cotransfected *AC8Luc*; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ as indicated.

4.5. Electrophoretic mobility shift assay (EMSA)

Nucleotide sequences of Ine and Pe1 described previously [88], and that of Dpe1 is presented later in Fig. 5A. Double-stranded oligonucleotides were synthesized for the Dpe1 element comprising positions −1879/−1791: 5'-GAG TCC AGT GTT TTC GTT TTT GGA GGC CCG GGG AA-3' (Dpe1A), 5'-GGA AAA ATT ATG TTT CAT ATA TTA AAA ATA AAC A-3' (Dpe1B), 5'-AAA TAA ACA CTA CTT TTA CAG AGG TAT AAA TGC-3' (Dpe1C). Coding region of Hmgb1 was inserted in frame into pGEX expression vector. GST-tagged L-Sox5, SOX9 and Hmgb1 were expressed and purified, and crude cell extracts were made as described [88]. 20-30 fmol end-labeled DNA probes were incubated either with 0.6-3.2 µg purified GST-fused Hmgb1, SOX9, L-Sox5 or 3 µg crude CEC or CEF cell extracts in the presence of 100-500 ng poly(dG-dC)·(dG-dC) and separated on pre-run 5% or 6.6% PAGE.

4.6. Immunofluorescence

Acetone-fixed 10-µm cryosections were used for immunofluorescence. Nonspecific binding

of the antibodies was blocked with 10% normal goat serum. The specimens were incubated at 4°C overnight with the following primary antibodies in combinations: rabbit affinity purified antisera specific for Matn1 [94, 95] (1:200 dilution) and SOX9 (Abcam, ab3697, 1:50) and mouse monoclonal antibody for HMGB1 (MBL, M137-3, 1:200). The appropriate secondary antibodies were applied at room temperature for 1 h in the dark: Alexa 488-labeled anti-rabbit IgG antiserum (Molecular Probes, 1:400), Cy3-conjugated anti-mouse IgG antibodies (Jackson Immunoresearch, 1:400). Nuclei were stained with 1 μg/ml Hoechst in PBS for 5 min. The specimens were mounted with fluorescent mounting medium (Dako), viewed with a Nikon Eclipse E600 microscope equipped with epifluorescence and Pan flour objectives, and photographed with Nikon digital camera D5000. After immunofluorescence, the coverslips were removed and the sections were restained with hematoxylin and eosin. The images were processed using SPOT software (version 4.0.9 for Windows; Diagnostic Instruments) and figures were made with Adobe Photoshop 8.0 and CorelDraw X4 softwares.

4.7. Forced expression assays combined with Western analysis and QRT-PCR

To estimate the relative expression levels of Sox and Nfi proteins, we used pcDNA5'UT-FLAG-SOX9 (pFSOX9) [52] and we made pFNfib by inserting the NotI (blunted)-NheI fragment of Nfi expression plasmids [63] into the EcoRI (blunted)-XbaI sites of pcDNA5'UT-FLAG. COS-7 cells were cotransfected as described above with *AC8Luc*, 1 μg pFSOX9 and increasing amounts of pFSox5 and pFSox6. In other experiments increasing amounts of pFNfib, effector plasmid was used with 1 μg pFSOX9. In addition, we made effector plasmid pFHmgb1 by inserting the Hmgb1 coding region [93] into pcDNA5'-UT-2FLAG. To estimate the relative expression levels of Sox and Hmgb1 proteins, we cotransfected COS-7 cells with 10 μg *AC8Luc*, 1 μg each of pFSOX9, pFSox5 and pFSox6, and increasing amounts of pFHmgb1. The transfected cells were lysed in 100 μl buffer containing 14 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 6 mM KCl, 0.44 mM NaCl, 0.08 mM EDTA, 3.5 mM DTT, 0.5 mM PMSF, 10% glycerol and protease inhibitor cocktail (Sigma-Aldrich P2714).

To test the induction of the endogenous *Matn1* in forced expression assays, we cotransfected COS-7 cells with 50 ng pFSOX9, 75 ng pFSox5, and 75 ng pFSox6 without and with 800 ng pFHmgb1 using 2μl TurboFect (ThermoScientific, R0531). Transfection mixtures were adjusted with empty vectors to the same amount of total DNA. Transfections were made in duplicates and repeated 3 times. RNA was isolated from the cells and the *Matn1* mRNA level was determined by QRT-PCR using the SybrGreen protocol and gene-specific primer pairs (Suppl. Table S2). C_τ values were normalized to that of Gapdh. Data are presented as mean ± SEM.

4.8. Hmgb1 silencing

Silencing experiments were performed in chondrogenic cell lines C-28/I2, SW1353, and RCS with siRNAs purchased from Bioneer Corporation (Daejeon, Republic of Korea) for human *HMGB1*: 5'-caggaggaauacugaacau-3'; for rat *Hmgb1*: 5'-cugucaacuucucagaguu-3'; for human *GAPDH*: 5'-gugugaaccaugagaagua-3', and for negative control siRNA: 5'-ccuacgccaccaauuucgu-3'. 1.2-2.0 x 10^5 cells were plated in 6-well plates and transfected with 100-400 pmoles of siRNA duplexes 24 h after plating using X-tremeGENE siRNA Transfection Reagent (Roche Applied Science) as suggested by the supplier. Cultures were harvested 30 h (RCS) or 42 h (C-28/I2, SW1353) after transfection. RNA was isolated from the cells and marker gene expression levels were determined by QRT-PCR using the SybrGreen protocol and gene-specific primer pairs (Suppl. Table S2). Gene expression levels were normalized to the invariant Rps18 mRNA levels. Data are presented as mean ± SEM from three independent experiments.

5. RESULTS

5.1. Accumulation of Nfi and Sox mRNAs during *in vitro* chondrogenesis

To address the potential contribution of Sox and Nfi factors and Hmgb1, we compared the kinetic changes in the expression of *Matn1*, Sox trio, Nfi and other marker genes in various chicken primary cultures by QRT-PCR during *in vitro* chondrogenesis in HDM culture (Fig. 4). This culture faithfully mimics the early steps of chondrogenesis as it differentiates to early proliferative chondroblasts characterized by elevated *Col6a1* expression (Fig. 4A). CEC culture, expressing the genes for Sox trio and cartilage proteins at high levels (Fig. 4A-C), represented a later stage. CEF culture served as a negative control. In CEF, the steady state mRNA levels for *Matn1* and the Sox trio were very low, while those for *Hmgb1* and Nfi except *Nfic* were elevated relative to their levels in committed mesenchyme (HDM day 0) (Fig. 4B-E).

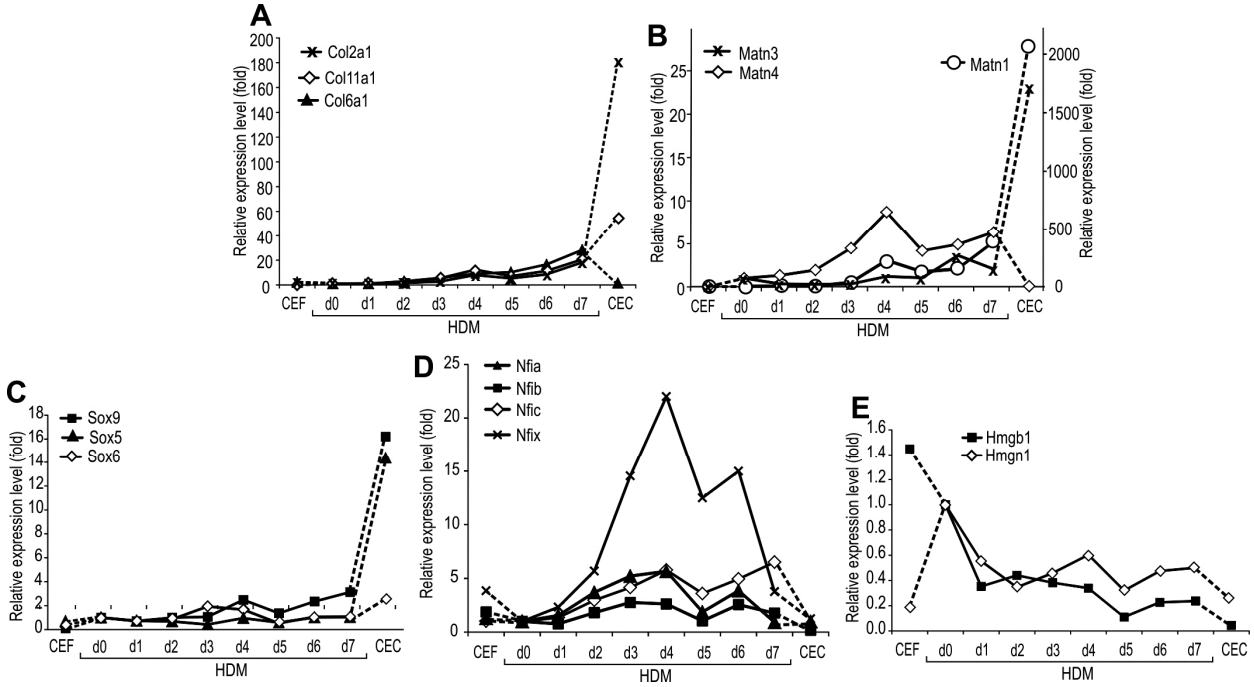

Fig. 4. Comparison of the marker gene expression by QRT-PCR during *in vitro* chondrogenesis. (A-E) Marker mRNA levels were determined by QRT-PCR in HDM culture undergoing chondrogenesis *in vitro* and compared to mRNA levels of non-expressing CEF and high *Matn1*-expressing CEC cultures. C_τ values were normalized to the average C_τ values of three internal control genes. Relative expression levels are presented as fold values relative to the HDM day 0 values.

Unlike the slow continuous accumulation of *Col2a1* mRNA during early stages of differentiation in HDM culture, activation of *Matn1* was first detected in HDM culture at day 4 (Fig. 4A and B). *Sox9* and *Col2a1* mRNAs accumulated with similar kinetics, but the low levels of L-Sox5 and *Sox6* mRNAs increased sharply only in CEC culture, except for a small, transient

boost of *Sox6* mRNA at days 3 and 4 in HDM culture, just preceding the first peak in the *Matn1* mRNA level (Fig. 4B and C). Interestingly, *Matn3* and *Matn4,* although also expressed in cartilage, exhibited smaller increase in their relative expression level than *Matn1* (Suppl. Table S1). *Matn4* level, however, peaked in HDM culture suggesting a function in early stage of chondrogenesis (Fig. 4B). Remarkably, from a very low expression level compared to the internal control genes measured in committed mesenchyme (Suppl. Table S1), *Matn1* expression showed the highest relative increase (2057-fold) in CEC culture, in contrast to the lower increase in the level of *Col2a1* (181-fold) and other cartilage ECM genes (<80-fold, compared to HDM day 0) (Fig. 4A and B). The relative Nfi mRNA levels also increased transiently 2.6 to 22-fold with two peaks at day 4 and days 6-7 in HDM culture, followed by a sharp decline in CEC culture close to 1 (*Nfia* and *Nfix*) or below 1 (*Nfib* and *Nfic*) (Fig. 4D). In contrast, Hmgb1 mRNA level declined gradually in HDM culture, exhibiting the lowest level in CEC culture (Fig. 4E). Thus, CEC culture, rich in late proliferative chondroblasts, is characterized by high *Matn1* and Sox trio, but low Nfi and Hmgb1 mRNA levels. Day 4 HDM culture, consisting of early proliferative chondroblasts, however, exhibits high Nfi, but lower *Matn1*, *Sox9* and *Sox6* mRNA levels and very low L-Sox5 mRNA expression. *Sox6* and Nfi mRNA levels peaked in HDM culture at the time of *Matn1* activation, suggesting a function in *Matn1* regulation.

5.2. Comparative binding of Sox transcription factors to the conserved *Matn1* promoter elements *in vitro*

It was reported previously that evolutionarily conserved *Matn1* DNA elements feature putative Sox motifs (Fig. 3) [86] and Pe1 and Ine were demonstrated to interact with SOX9 *in vitro* [86, 88, 89]. Therefore, we hypothesized that Sox factor-mediated interactions may play important role in the chondroblast developmental stage-dependent regulation of *Matn1*. To test this hypothesis, first we compared the binding of purified chondrogenic Sox factors to Dpe1 and the short promoter elements Pe1 and Ine (Fig. 5).

We found that the chicken Dpe1 element harbors three putative sites with tandem and inverted paired motifs, which share 5/7 or 6/7 nucleotide identity with the Sox consensus sequence (Fig. 5A). These motifs also show 5/10 to 8/10 nucleotide identity with AGAACAATGG, the preferred Sox9-binding site [96]. We performed EMSA experiments to demonstrate that the element carries at least three sites which can interact with purified Sox proteins *in vitro*. Each of the three subfragments of Dpe1 was clearly recognized by GST-fused SOX9 and L-Sox5, but with inverse binding efficiency (Fig. 5B and C). SOX9 exhibited the strongest binding to Dpe1C forming two complexes and weaker binding to Dpe1A and Dpe1B

forming one and three diffuse complexes, respectively (Fig. 5B). On the other hand, L-Sox5 most preferably recognized Dpe1B, followed by A and C (Fig. 5C). We found that SOX9 bound the *Matn1* control elements with highly variable efficiency. Whereas it showed more potent complex formation *in vitro* with each of the Dpe1 Sox sites, than with those of Ine, SOX9 bound Pe1 even more powerfully (≥5-fold) than Dpe1C (Fig. 5B).

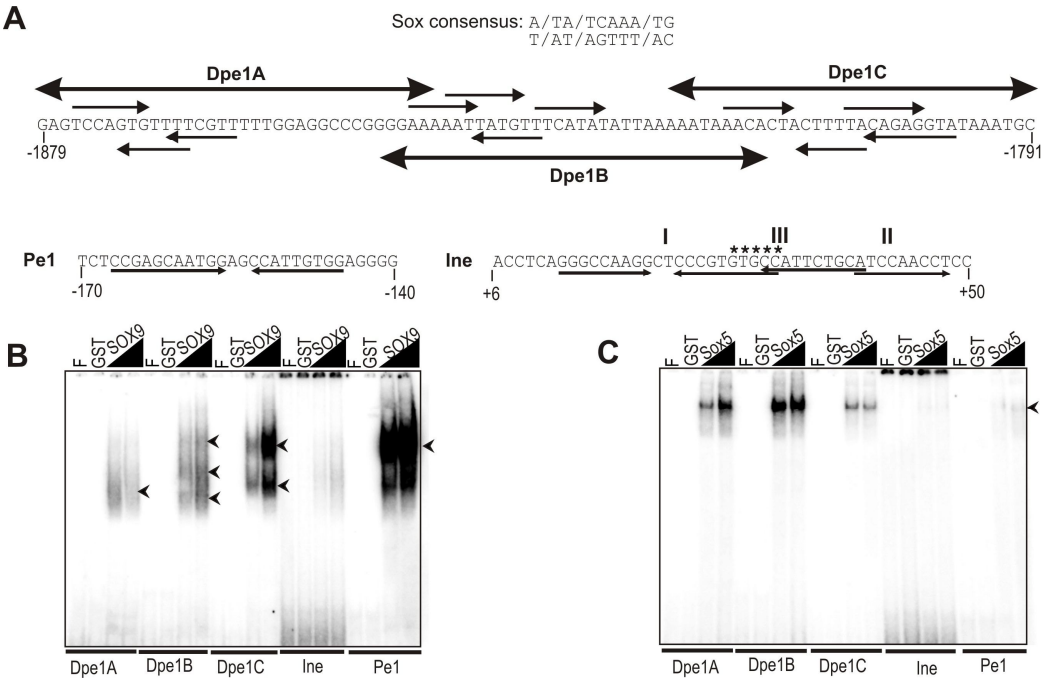

Fig. 5. Comparison of binding purified GST-fused Sox proteins to the conserved DNA elements. (A) Nucleotide sequences of Dpe1, Pe1 and Ine elements. Large arrows mark the subfragments of Dpe1 and small arrows depict the Sox motifs of the elements. The conserved GTGCC motif the 5' (I) and 3' (II) paired Sox sites and unrelated factor-binding site (III) of Ine are denoted. Comparison of binding of GST-fused SOX9 (B) and L-Sox5 (C) to the Dpe1 subfragments, Ine and Pe1 in EMSA. F, free probe.

Pe1, the most conserved DNA element in amniotes bears a palindrome very similar to the preferred Sox9-binding site (Fig. 3B) [86, 96]. This motif was recognized by the Sox trio *in vitro* and protected in genomic footprinting in our laboratory [86]. Consistent with these previous findings, we observed that as opposed to purified L-Sox5, SOX9 binds most efficiently Pe1 among the conserved *Matn1* elements (Fig. 4B and C), strongly suggesting that Pe1 likely plays a key role in Sox-mediated interactions. Conserved sequences with two pairs of inverted Sox motifs were also found in the Ine element of mammals and the sequence was less conserved between mammals and chicken, but the chicken sequence also featured two paired Sox motifs around the transcription start site (Fig. 3A) and [97]. A conserved GTGCC motif in Ine, and an Nfi site upstream of TATA were also found. In agreement with other observations in our

laboratory [88], Ine interacted with SOX9 and L-Sox5 with low efficiency (Fig. 5B and C). SOX9 and L-Sox5 bound somewhat more strongly to the 5' and 3' Sox-binding sites, respectively. In fact, purified Sox factors bound the 5' and 3' Sox sites of Ine cooperatively in EMSA [88].

Interaction of Dpe1 with SOX9 and L-Sox5 *in vitro* supports the hypothesis that it may exert enhancement via binding Sox factors. The strongest interaction of Pe1 with SOX9 suggests that element plays a crucial role in SOX9-mediated transactivation from the upstream elements. The L-Sox5/Sox6 bound to Ine may modulate transactivation by SOX9. Interaction of Ine with Sox factors was the weakest among the elements.

5.3. The Pe1 Sox site and SI Nfi site are indispensable for promoter activation in transiently transfected chondrocytes

To study the contribution of conserved DNA elements to promoter activity, we introduced point mutations into Ine, Pe1, and Dpe1 and measured their effect on the activity of long promoter constructs (*AC8Luc*) (Fig. 6A and B). As the SI element is also conserved in the short *Matn1* promoter of amniotes (Fig. 3A) [97] and it is recognized by Nfi proteins *in vitro* and *in vivo* [86, 89], we similarly studied that effect of SI mutation on the long promoter activity. The −2011/−334 sequence enhanced the short promoter activity ~19-fold in *AC8Luc* in CECs, but hardly did so in low- or non-expressing cultures (Fig. 6A). Sox site mutations IneM1, IneM2 and IneM3 (Fig. 6B) cut the long promoter activity to half or more in CECs. The effect of Pe1M4, which carried a mutation unrelated to Sox binding site [86], was similar, but Pe1M1, in which the Sox site of Pe1 was disrupted, dropped the long promoter activity 13-fold, abolishing CEC-specific enhancement from upstream elements (Fig. 6A). The double mutant Pe1M1/IneM2 disrupting all the three Sox- binding sites in the short promoter decreased the long promoter activity ($p<0.05$) even closer to that of *FO15Luc*. Thus, the Sox sites in Pe1 and Ine are needed to mediate promoter activation from upstream elements. Deletion of the entire Dpe1 element or its subfragments B and C decreased the long promoter activity by 4-fold and 2-fold, respectively, in CEC culture, while deletion of Dpe2 had milder effect (data not shown).

Considering that the SI element was protected in genomic footprinting in CEC culture [86, 89] and bound Nfi proteins in vitro [87], we also mutated its Nfi contact points. Mutation SI2dm either alone or in combination with Pe1M1 or Pe1M4 dropped the long promoter activity by 10-fold in CEC and similarly in other cultures indicating tissue-unspecific inhibition (Fig. 6A). Double mutation Pe1M1/SI2dm further diminished the activity ($p<0.001$) to the basal promoter level in mesenchymal cells suggesting an additive or synergistic effect. Thus, disruption of the

Nfi site of the SI silencer element abolished both the tissue- and stage-specific promoter activity.

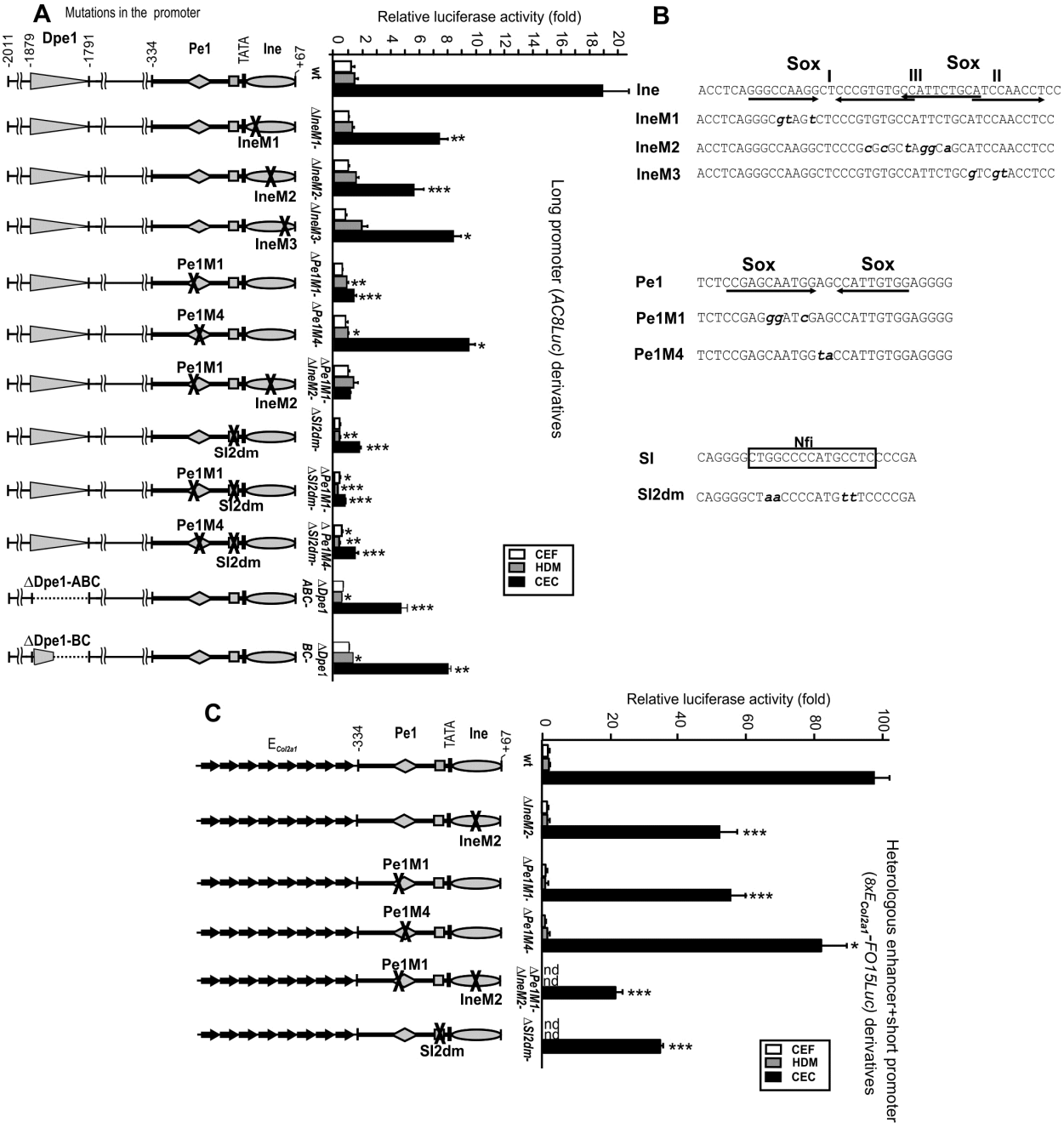

Fig. 6. Effect of Ine, Pe1 and SI mutations and Dpe1 deletion on the long promoter activity in mesenchymal cultures. Transient expression activities of wild-type and mutant versions of reporters *AC8Luc* (A) and *8xE$_{Col2a1}$-FO15Luc* (C) driven by long *Matn1* promoter or multiple E$_{Col2a1}$ fused to the short promoter, respectively. Schematic to the left indicate single or double mutations introduced into the promoter (B) Sequences of wild-type or mutant version of DNA elements. Sox- and Nfi-binding sites are indicated (A and C). Luciferase activities of wild-type (wt) and mutant reporters in the low-, high-, and nonexpressing HDM, CEC, and CEF cultures, respectively, are presented in fold relative to that of *FO15Luc*. *p<0.05, **p<0.01, ***p<0.001 mutants vs. wild-type reporters.

We concluded that albeit Ine recognition by Sox factors may be involved, Sox factor binding to Pe1 seems to be more crucial for promoter activation in CEC culture rich in late proliferative chondroblasts. However, the short promoter elements alone direct only a low reporter gene activity and promoter upstream elements are needed for its enhancement. We found that Dpe1 is a very important upstream element necessary for the activation of the short promoter. These data are consistent with the high enhancement of the short promoter by multiple copies of Dpe1 in CEC culture and transgenic mice [88]. In addition, binding of the ubiquitous Nfi to SI near the TATA box may be similarly crucial. The position-specific conservation of motifs similar to the NFI consensus [98] near TATA in amniotes [88] further supports the importance of SI in the regulation of the gene. The significant, but less dramatic effect of other mutations suggests that binding of factors to the Pe1 spacer and to the conserved motif of Ine may also be needed for full promoter activity.

5.4. Sox and Nfi sites of the short promoter are important for enhancement by a heterologous cartilage-specific enhancer.

Next we tested the activation of the short promoter by a heterologous cartilage enhancer. Toward this end we chose multiple copies of the *Col2a1* enhancer element (E_{Col2a1}), which is known to function in chondrocytes independent of their differentiation stages [78]. Eight copies of E_{Col2a1} robustly increased the short promoter activity in CEC, but had no effect in CEF or in HDM cultures consisting of early proliferative chondroblasts (Fig. 6C). Lining up with transgenic mouse data [88], these results indicate that the *Matn1* short promoter also restricted the broad cartilage-specific enhancement by E_{Col2a1} to late proliferative chondroblasts in tissue culture.

Mutations Pe1M1, IneM2, SI2dm and Pe1M1/IneM2 decreased the relative activity of *8x*E_{Col2a1}*-FO15Luc* by 43.6%, 46.6%, 64.9%, and 78%, respectively, in CEC culture (Fig. 6C). Thus, our data show that whereas Sox factor binding to Pe1 is crucial for the interaction between the homologous distal and proximal DNA elements, Sox9 binding to Pe1 and Ine is less essential for mediating enhancement from E_{Col2a1}. Disrupting all Sox sites of Pe1 and Ine or the Nfi site of SI, however, highly diminished the enhancement, supporting the hypothesis that the short promoter elements may also interact with the heterologous enhancer via the bound Sox and Nfi factors.

5.5. Dose-dependent synergy of L-Sox5/Sox6 with SOX9

Next we assessed the activation of the *Matn1* long promoter by cotransfected Sox proteins (Fig. 7A-D). While SOX9 doubled it, L-Sox5/Sox6 decreased the promoter activity to about half

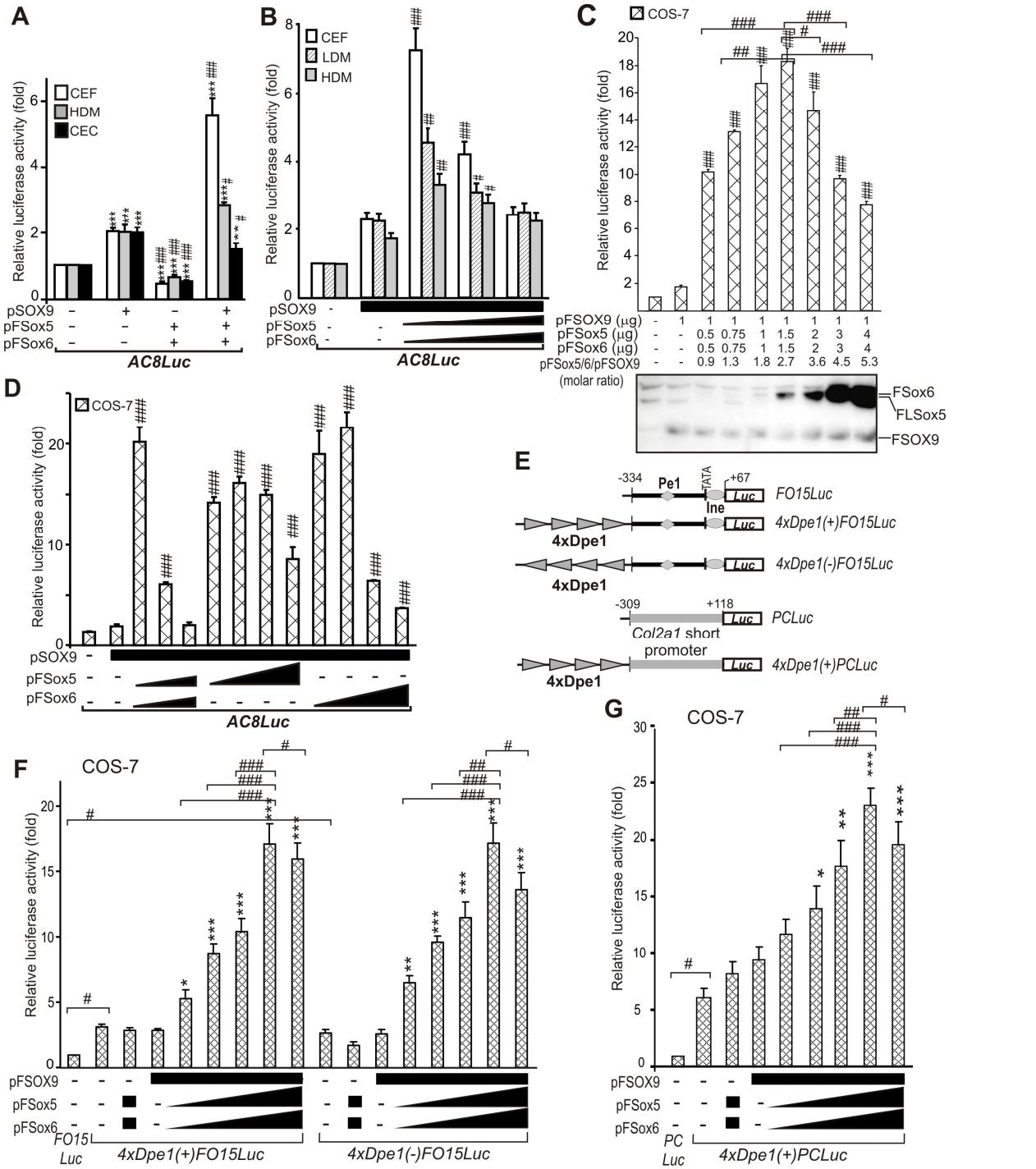

Fig. 7. Dose-dependent activation of the *Matn1* promoter and Dpe1 fused to homologous or heterologous short promoters by the Sox trio. (A-D) *AC8Luc* was cotransfected with Sox expression plasmids in various mesenchymal cultures as indicated (A-B) and COS-7 cells (C-D). Combined forced expression assay and Western analysis with anti-FLAG antibody (C). Forced expression assays were performed with constant amount of FLAG-tagged expression plasmid for SOX9 and increasing amount of expression plasmids for L-Sox5 and Sox6. (E) Map of the reporters driven by four copies of Dpe1 fused to the homologous *Matn1* or the heterologous *Col2a1* short promoters in direct or reverse orientations as indicated. (F-G) Dose-dependent synergistic activation of these reporters by forced expression of the Sox trio. Luciferase activities are presented as fold values relative to that for *AC8Luc* (A-D), *FO15Luc* (F) and *PCLuc* (G). *p<0.05, **p<0.01, ***p<0.001 compared with reporter cotransfected with vectors (A-D) or vs. vector-cotransfected 4xDpe1-reporters (F-G); $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 compared with SOX9 cotransfected reporters (A-D) or as indicated (C,F and G).

in mesenchymal cells (Fig. 7A). Coexpression of L-Sox5/Sox6 with SOX9 greatly or moderately increased the ability of SOX9 to activate the promoter in CEF and HDM cultures, respectively, but rather decreased it in CEC culture. This indicates synergy between Sox proteins at early differentiation stages. When we introduced constant amount of pSOX9 and increasing amounts of pSox5 and pSox6 expession plasmids into CEF, LDM and HDM cultures, the synergistic activation peaked at low ratio of pSox5 and pSox6 versus pSOX9, and declined at elevated ratio to the level achieved by SOX9 alone (Fig. 7B). Highest activation was seen in CEF (3.5-fold), followed by that in LDM and HDM cultures in inverse correlation with the endogenous *Sox5* and *Sox6* expression levels of these cultures (Fig. 4C), raising the possibility that L-Sox5/Sox6 may modulate the activation by SOX9 in a dose-dependent manner.

We confirmed this hypothesis by forced expression of FLAG-tagged Sox trio in nonchondrocytic COS-7 cells and monitoring the protein expression in Western blots (Fig. 7C). Despite the low effect of SOX9 alone, L-Sox5/Sox6 synergized with SOX9 to activate the long promoter up to ~18-20-fold at low molar excess. The activation was high from 0.9:1 to 3.6:1 molar ratio of L-Sox5/Sox6 to SOX9 in repeated experiments, but the synergy dropped above 5.3:1 molar ratio (Fig. 7C). L-Sox5 and Sox6 had similar effect with sharper decline for Sox6 (Fig. 7D).

We concluded that L-Sox5/Sox6 can finely tune the *Matn1* promoter by greatly increasing the ability of SOX9 to activate the promoter at low molar excess to SOX9 (early stages of chondrogenesis), or decreasing the activation by SOX9 at high excess (late stage), possibly by competing with SOX9 for its binding sites.

5.6. Dpe1 can function as a cartilage-specific enhancer element in cotransfection experiments in culture

Next we assessed, if Dpe1 elements can exert Sox trio-mediated enhancement to homologous and heterologous promoters in COS-7 cells forced to express constant amount of SOX9 and increasing amounts of L-Sox5 and Sox6 (Fig. 7E-G). Four copies of Dpe1 increased the activity of the *Matn1* and the *Col2a1* promoters by 3.1- and 6-fold, respectively, which was not or only slightly increased further by forced expression of SOX9 or L-Sox5/Sox6 alone (Fig. 7F and G). L-Sox5/Sox6, however, synergized with SOX9 in a dose-dependent manner to mediate a 17- and 23-fold activation of the *Matn1* and *Col2a1* short promoters, respectively, from the Dpe1 elements. As the Dpe1 elements worked efficiently in both orientations (Fig. 7F) and enhanced even the heterologous *Col2a1* promoter (Fig. 7G), we concluded that Dpe1 can function as a cartilage-specific enhancer element.

5.7. Pe1 mutation and Dpe1 deletion hampers the transactivation by SOX9, Ine mutants decrease the synergism with L-Sox5/Sox6

Next we studied the effect of Pe1, Ine and SI mutations and Dpe1 deletion on the SOX9- and Sox trio-mediated enhancement in coexpression assays (Fig. 8A). In COS-7 cells forced to express L-Sox5/Sox6 in optimal abundance to SOX9, Pe1M1/IneM2 decreased the synergistic activation of the long promoter by 96.1%, followed by SI2dm and Pe1M1 (85.5-89%) (Fig. 8A and B). Pe1M1/IneM2 and SI2dm also repressed the SOX9-mediated activation by ~70%. Pe1M1/IneM2 similarly decreased the SOX9 and/or Sox trio mediated activation by 70-96%, when L-Sox5/Sox6 was coexpressed in higher excess to SOX9 in LDM, and CEC cultures (Fig. 8C and D). Thus disruption of all the three Sox sites of the short promoter abolished the transactivation by SOX9 and the Sox trio even in the presence of intact upstream elements. The effect of Pe1M1 was only somewhat milder, demonstrating that SOX9 binding to Pe1 is critical for the SOX9-mediated promoter activation at both early and late stage of chondrogenesis.

Ine mutations diminished the synergistic activation of SOX9 by optimal excess of L-Sox5/Sox6 in COS-7 cells (Fig. 8A and B). In LDM culture, IneM1 and IneM3 also abolished the activation of *AC8Luc* by SOX9, whereas IneM3 and IneM2 more drastically diminished the synergistic effect of elevated L-Sox5/Sox6 than IneM1 (Fig. 8C). In keeping with the effect of Ine mutations in EMSA [88], this indicates that even though Sox factors bind both the 5' and 3' Sox sites of Ine (Fig. 5D-F), the 3' site equally interacts with SOX9 and L-Sox5/Sox6, but the 5' site is preferably recognized by SOX9 in early proliferative chondroblasts. IneM1 also hampers the activation by SOX9 in CEC culture (Fig. 8D). Notably, mutation of the Nfi site of SI also abolished or highly decreased the SOX9- and Sox trio-mediated promoter activation in the cultures tested (Fig. 8). Variable effect of Pe1M4 mutation and the low effect of SOX9 in COS-7 cells indicate that ubiquitous and/or Sox partner factors may also bind the promoter elements.

Deletion of Dpe1 decreased ~3-fold the transactivation of the promoter with SOX9 in LDM culture and close to half in CEC culture (Fig. 8C-D). It also decreased to half the synergistic activation by the Sox trio in LDM culture or in COS-7 cells forced to express L-Sox5/Sox6 in optimal dose relative to SOX9, but not in CEC culture expressing the Sox trio at high level (Fig. 8).

We concluded that SOX9 binding to Pe1 plays key role in mediating the enhancement from distal elements. L-Sox5/Sox6 expressed in low relative abundance and bound to Ine can synergistically increase the activation by Pe1-bound SOX9 at early stage of chondrogenesis. At late stage, however, when produced in large excess to SOX9, L-Sox5/Sox6 may decrease the

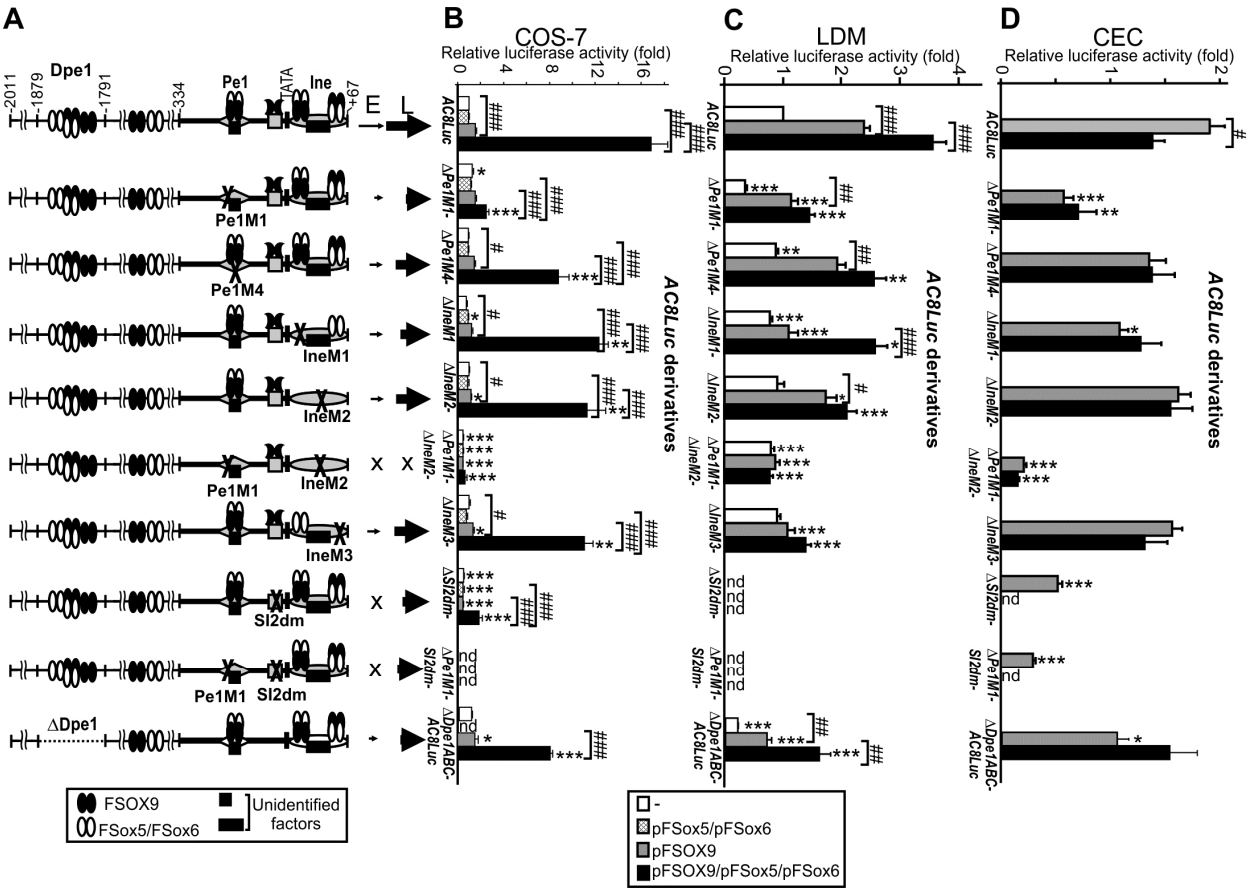

Fig. 8. Effect of point mutations on the synergistic activation of the long promoter by the Sox trio in cultures. (A) Schematic indicates factor binding to the short promoter and upstream element (Dpe1). Thin and thick arrows depict the transcription efficiency at early (E) and at late stage (L) of chondrogenesis. (B-D) Activation of wild-type and mutant reporters by L-Sox5/Sox6 and SOX9 coexpressed at optimal (2.7:1) molar ratio in COS-7 cells (B) and at higher ratio in LDM (C) and CEC (D) cultures. Luciferase activities are given in fold relative to that of *AC8Luc*. *p<0.05, **p<0.01, ***p<0.001 mutants vs. similarly cotrasfacted *AC8Luc* (B-D); $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 as indicated (B-D).

activation by SOX9, possibly by competing for its binding sites. In addition, Nfi binding to SI and other factor binding to Pe1 and Ine is also needed for efficient promoter activation. Based on deletion analysis, SOX9 binding to Dpe1 plays an important role in promoter activation in late proliferative chondroblasts, possibly via Sox-mediated interactions with Pe1. In early steps of chondrogenesis, however, SOX9 binding to Dpe1 as well as synergistic activation of SOX9 by an optimal dose of L-Sox5/Sox6 is also needed for promoter enhancement.

5.8. Nfi proteins modulate the promoter activity

Next we studied the effect of Nfi on *AC8Luc* activity in cotransfection assays. In CEC culture, all Nfi proteins, except Nfia at low concentration, robustly inhibited the long promoter activity (Fig. 9A). When Nfi and SOX9 were expressed at an optimal ratio, Nfib and Nfic

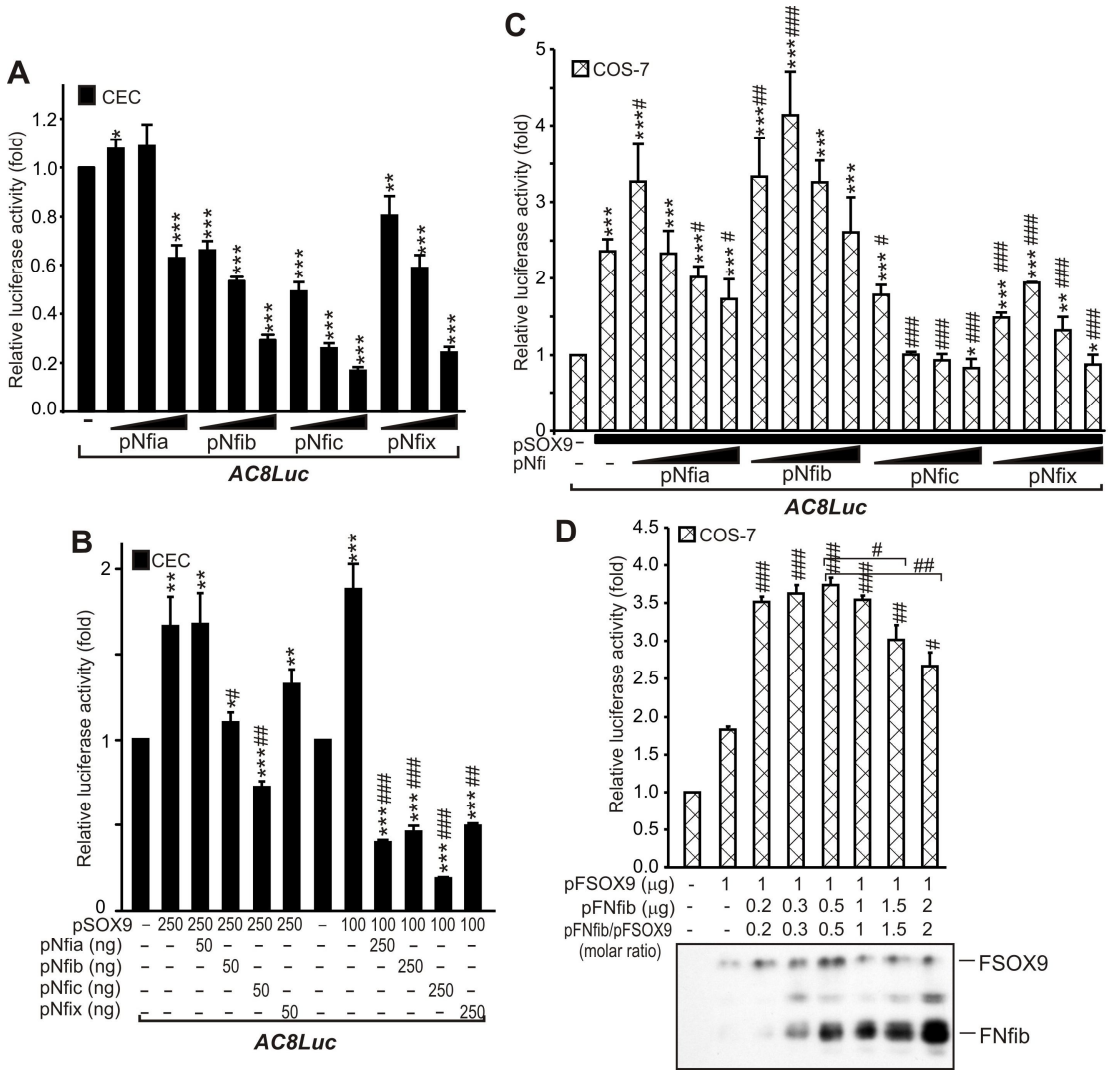

Fig. 9. Modulation of the *Matn1* promoter activity by cotransfected Nfi proteins. (A-D) *AC8Luc* was cotransfected with increasing amount of Nfi expression plasmids without or with constant amount of SOX9 expression plasmids in cultures as indicated. (D) Combined forced expression and Western analysis in COS-7 cells. Flag-tagged SOX9 and Nfi proteins were detected by Western analysis using anti-FLAG antibody to determine the relative ratio of Nfib and SOX9 expressed by force at optimal promoter activation.

decreased significantly the transactivation by SOX9, but all Nfi proteins exerted 74% to 90% repression at higher ratio (Fig. 9B). In COS-7 cells, Nfia and Nfib in optimal amount indeed cooperated with SOX9 and potentiated its transactivation of the long promoter (Fig. 9C). The activation, however, declined at higher levels of Nfia and Nfib. In contrast, Nfic and Nfix inhibited the activation by SOX9. Forced expression of FLAG-tagged proteins in COS-7 cells revealed that the activation increased up to ~2:1 molar ratio of Nfib to SOX9, but significantly decreased above ~4:1 molar ratio (Fig. 9D).

These data suggest that Nfi proteins may increase or decrease SOX9-mediated

transactivation of *Matn1* depending on their abundance relative to SOX9 (see later Fig. 15A). The conservation of Nfi sites near TATA and Pe1 [88] underlines the importance of Nfi proteins in the restricted cartilage-specific expression of *Matn1* in amniotes.

To sum up, our data suggest a model of the developmental-stage specific regulation of *Matn1* (see later Fig. 15A). This model involves unique arrangement of conserved DNA elements. Short promoter elements Pe1 and Ine bind preferentially to Sox9 and L-Sox5/Sox6, respectively; and SI and SII elements are recognized by Nfi proteins. The promoter upstream enhancer element Dpe1 interacts with the Sox-trio. Developmental stage-specific regulation is achieved, as binding and activity of the Pe1-bound Sox9 is dose-dependently modulated by the Ine-bound L-Sox5/Sox6 proteins. Dpe1 enhances the promoter activity via Sox-mediated interaction with Pe1.

5.9. *Hmgb1* is expressed in early chondrogenesis in inverse correlation with chondrogenic markers

Sox proteins and Hmgb1 have similar HMGB domains and Hmgb1 secreted by hypertrophic chondrocytes was implicated in late steps of endochondral ossification [8], but its function in early steps has not been studied yet. To address the possible involvement of Hmgb1 in chondrogenesis, we used double immunofluorescence to monitor Hmgb1 expression in the developing limbs of mouse embryos (Fig. 10). We observed that the ubiquitous Hmgb1 immunosignal started to decrease in early steps of chondrogenesis, showing overlap with Sox9 in condensed mesenchyme, prechondrocytes and early chondroblasts (Fig. 10A, B, and D). In line with former data [3, 20, 57], Matn1 had a narrower spatiotemporal expression pattern than Sox9, being first detectable only in early chondroblasts with some delay (Fig. 10C and E). Thus only a very limited overlap was seen between Hmgb1 and Matn1 in the latter cells at the onset of *Matn1* (Fig. 10E). Hmgb1 expression, however, dropped as chondrogenesis progressed, exhibiting a complementary pattern to that of Sox9 (Fig. 10B and D) and Matn1 (Fig. 10C and E) in overtly differentiated cartilaginous elements.

These results are consistent with the kinetic changes in the marker gene expression we revealed by QRT-PCR in HDM culture undergoing chondrogenesis *in vitro* (Fig. 4E). The Hmgb1 mRNA level was highest in CEF culture, it declined gradually during differentiation in HDM culture and it was the lowest in CEC culture. Down-regulation of *Hmgb1* during chondrogenesis was more pronounced than that of *Hmgn1* known to activate *Sox9* [99]. Both *Hmgb1* and *Hmgn1* levels showed an inverse correlation with those of *Sox9, Sox5* and most of the cartilage ECM genes (*Col2a1*, *Col11a1*, *Matn1*, and *Matn3*) (Fig. 4A-C and E). At day 4 when *Matn1* was first detected in HDM culture, the sharply declining Hmgb1 mRNA level

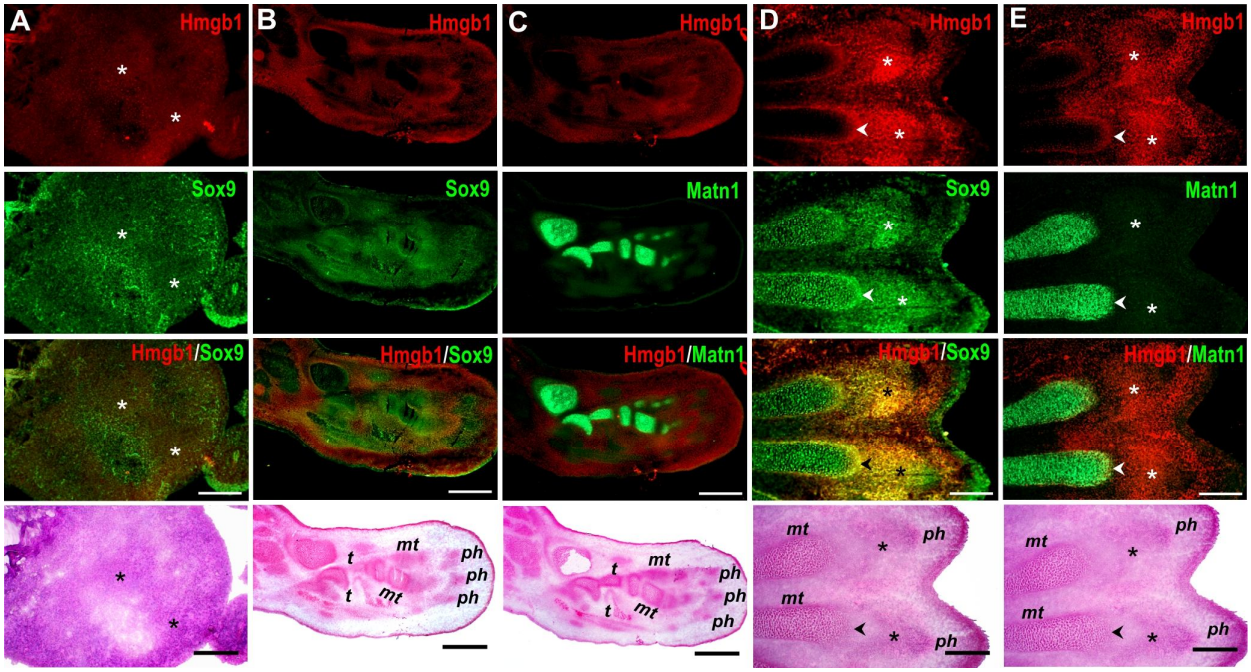

Fig. 10. Expression of *Hmgb1* is complementary to those of *Sox9* and *Matn1* in the developing mouse limb. Double immunofluorescence on consecutive cryosections of E12 (A) and E14.5 mouse embryos (B-E). Hematoxylin-eosin staining of the same cryosections is shown below for comparison. The expression domains of Hmgb1 and Sox9 overlap with each other in early steps of chondrogenesis just before and at the time of activation of *Matn1*. Note the overlapping Hmgb1 and Sox9 signals e.g. in condensed mesenchyme or prechondrocytes (asterisk) and early chondroblasts of developing metarsals (arrowhead) (A, B and D), which exhibit no or yet low Matn1 immunosignal, respectively (C and E). In the overtly differentiated cartilaginous elements, however, the Hmgb1 signal ceases, complementary to the high Sox9 and Matn1 signals (B-E). Bars, 200 µm (A, D and E); 500 µm (B and C). mt, metatarsals; ph, phalanges; t, tarsals.

($1.5x10^{-3}$) relative to the internal control genes was only 2.3-fold higher than the increasing Sox9 mRNA level ($6.6x10^{-4}$) (Suppl. table S1). This ratio decreased to 1.3 by day 7 in HDM culture. In CEC culture consisting mostly of late proliferative (stage Ib) chondroblasts, however, the mRNA levels for Sox9 and L-Sox5 were 21.5-fold and 15.5-fold higher, respectively, than that for Hmgb1.

To sum up, *Hmgb1* expression gradually declined in an inverse correlation with the activation of the chondrogenic Sox and ECM genes during limb development and in chondrogenic cultures, exhibiting a small overlap in their expression domains in early steps of *in vivo* and *in vitro* chondrogenesis. Coexpression of *Hmgb1* and *Sox9* at comparable levels just before and at the time of the onset of *Matn1* raises the possibility that, in addition to Sox9, Hmgb1 may also contribute to the gene regulation in early steps of chondrogenesis.

5.10. Hmgb1 increases the *Matn1* promoter activity in cotransfection assays in early stages of chondrogenesis

Next we assessed whether Hmgb1 can influence the *Matn1* promoter activity in cotransfection assays. We observed transient activation of the long promoter by increasing amount of pHmgb1 expression plasmid with the highest peak (2.6-fold) in HDM culture and a lower increase in LDM and CEF cultures (Fig. 11A). The small transient increase, however, turned to inhibition at higher doses of Hmgb1 in CEC culture. Thus, Hmgb1 can markedly increase the long promoter activity in mesenchymal cultures, where the Sox trio expression is low and the *in vivo* occupancy of Sox-binding sites of Pe1 and Ine is very limited [86, 89]. In CEC culture, however, where the Sox trio expression is high and Pe1 and Ine are occupied by *in vivo* bound factors [86, 89], elevated amount of Hmgb1 does not activate, but rather inhibits transcription. Therefore, we hypothesized that Hmgb1 acting as an architectural protein may increase the promoter activity in early steps of chondrogenesis and facilitae the synergistic activation by the Sox trio.

To test this hypothesis, we measured the long promoter activity by coexpressing Sox proteins with optimal amount of Hmgb1. In increasing dose, SOX9 gradually increased, whereas L-Sox5/Sox6 gradually decreased the promoter activity in mesenchymal cultures (data not shown). When constant amount of SOX9 and increasing amount of L-Sox5/Sox6 were coexpressed with and without optimal amount of Hmgb1, Hmgb1 exerted only a small significant increase in the transactivation by SOX9, but it doubled the dose-dependent synergistic activation of SOX9 by L-Sox5/Sox6 in LDM and HDM cultures, by compensating the decline caused by higher doses of L-Sox5/Sox6 relative to SOX9 (Fig. 11B). In CEF culture, Hmgb1 activation (2.1-fold) peaked at optimal dose of L-Sox5/Sox6 versus SOX9, thus raising the promoter activity up to 9.3-fold (Fig. 11B and C).

To further prove that Hmgb1 can facilitate the synergistic promoter activation by the Sox trio, we forced to express constant amount of Sox proteins and increasing amount of Hmgb1 in COS-7 cells and monitored the expression of the FLAG-tagged proteins in Western blots (Fig. 11D). We found that the Sox trio-mediated 17.6-fold enhancement of the promoter activity increased further to 38-fold at ~3:1 molar ratio of Hmgb1 to SOX9.

We concluded that optimal dose of Hmgb1 can facilitate the dose-dependent synergistic activation of the *Matn1* promoter by the Sox trio in early chondrogenesis or in cultures expressing the Sox trio at a low level. The activation, however, turns to inhibition in CEC culture exhibiting high endogenous Sox mRNA levels.

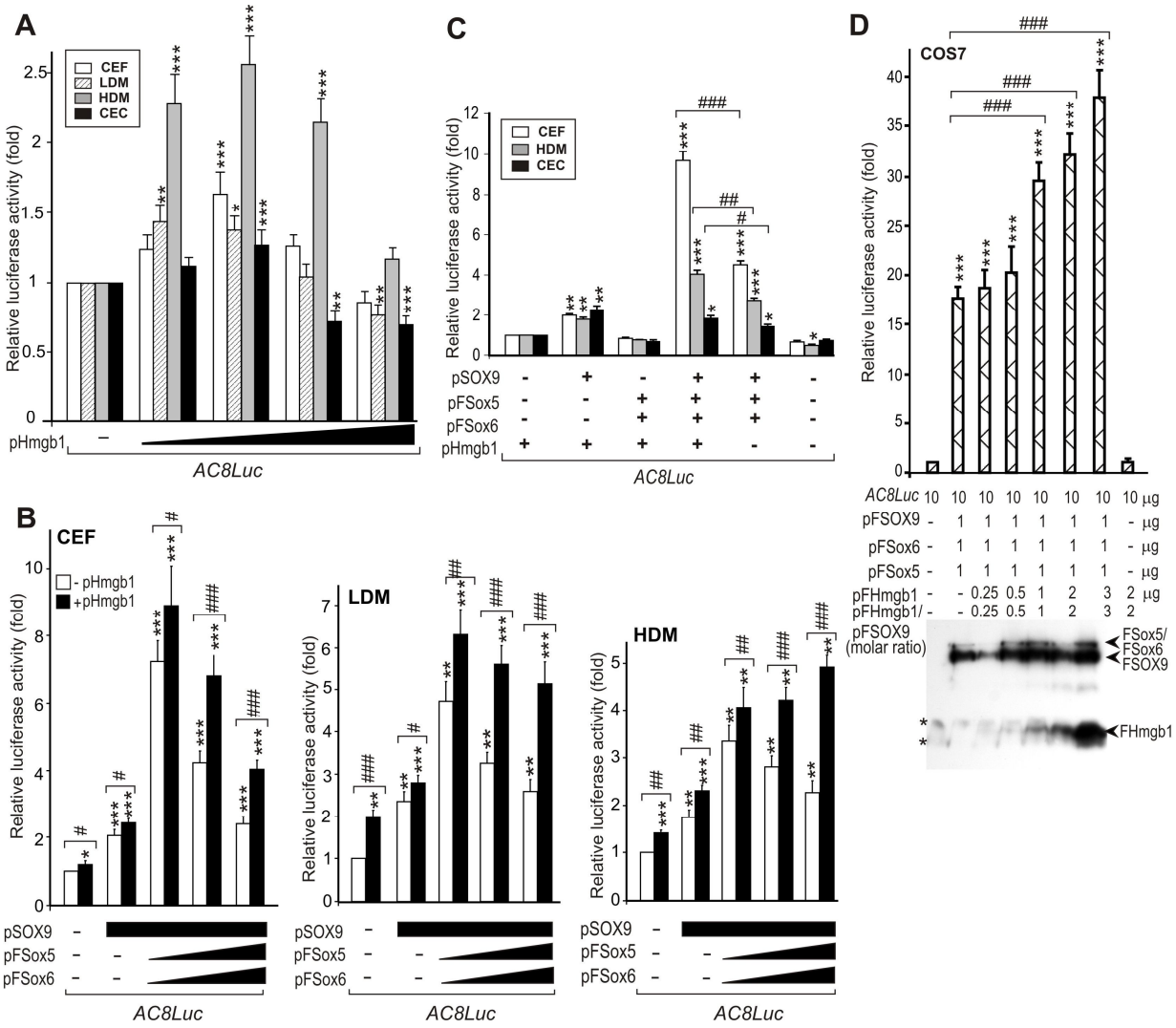

Fig. 11. Effect of Hmgb1 on the synergistic activation of *Matn1* promoter by the Sox trio. (A) Transient activation of the long promoter by Hmgb1 in cotransfection assays. (B- C) Effect of coexpressed Hmgb1 and Sox trio on the long promoter activity in mesenchymal cells. (D) Forced expression of increasing amount of FLAG-tagged Hmgb1 and constant amount of Sox trio in COS-7 cells combined with Western analysis using anti-FLAG antibody. Nonspecific bands are marked with asterisk. Luciferase activities are presented as fold values relative to that for *AC8Luc* (A, B and D) or for Hmgb1-cotransfected *AC8Luc* (C). *p<0.05, **p<0.01, ***p<0.001 vs. vector-cotransfected reporter (A-D); $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 as indicated (B-D).

5.11. Hmgb1 facilitates activation of the endogenous Matn1 in forced expression assays

Next we performed forced expression experiments in COS-7 cells to test whether optimal levels of the chondrogenic Sox proteins are sufficient for ectopic induction of the chromosomal *Matn1* and whether Hmgb1 can enhance the induction. As shown in Table 1, forced expression of the Sox trio in optimal L-Sox5/Sox6 dose relative to Sox9 increased the very low expression level of the endogenous *Matn1* by 26-fold. Forced expression of Hmgb1 did not have a

significant effect alone, but together with the Sox trio it increased the *Matn1* expression level by 79-fold as compared to the vector-transfected control. This confirms the ectopic induction of the endogenous *Matn1* by forced expression of Sox trio in the nonexpressing COS-7 cells and an additional 3-fold activation by forced expression of Hmgb1. We concluded that Hmgb1 can facilitate not only the Sox trio-mediated activation of the reporter gene driven by the long *Matn1* promoter (Fig. 11D), but it can also increase activation of the chromosomal *Matn1* by the Sox trio.

Table 1. Induction of the endogenous *Matn1* in the nonexpressing COS-7 cells by forced expression of the Sox trio and Hmgb1

Transcription factors expressed by force	*Matn1* expression level $2^{-\Delta C\tau}$±SEM[a]	fold	p value
Empty Vector	1.95E−06 ± 1.47E−07	1	
Hmgb1	2.42E−06 ± 2.87E−07	1.24	9.90E−01
Sox trio	5.03E−05 ± 5.64E−06	25.76	7.08E−03
Sox trio + Hmgb1	1.54E−04 ± 2.65E−05	78.68	2.25E−05

[a]*Matn1* mRNA levels are given relative to the invariant Gapdh mRNA level.

5.12. Hmgb1 forms nucleoprotein complexes on the *Matn1* promoter elements *in vitro*

We next studied the *in vitro* interaction of the conserved DNA elements with GST-fused purified Hmgb1. In inverse correlation with their SOX9-binding efficiency, Dpe1A and Dpe1B bound more strongly whereas Pe1 and Dpe1C bound weakly to Hmgb1 (Fig. 12A). Ine showed weak interaction with both proteins. When Hmgb1 was added in increasing amount together with Sox factors, it modulated the binding of Sox factors to the elements in a dose-dependent manner (Fig. 12B and C). Curiously, it decreased the binding of SOX9 and L-Sox5 to Dpe1A or, after an inhibition at a low dose, it transiently increased the formation of L-Sox5 and SOX9 complexes on Dpe1B at higher doses of Hmgb1 relative to Sox factors. In contrast, Hmgb1 increased the binding of Dpe1C, Ine and Pe1 to SOX9 and L-Sox5 at an optimal dose, followed by a decrease at higher dose. Hmgb1 also modulated the binding efficiency of SOX9 in the presence of L-Sox5: either decreased both (Ine) or dose-dependently increased the formation of SOX9-specific complexes (Pe1 and Dpe1C) or transiently increased the formation of L-Sox5 complexes, while transiently inhibited the SOX9 complexes (Dpe1A and Dpe1B). Pe1M1 mutation disrupting the paired Sox9 motifs, but not the palindrome structure of Pe1, extremely reduced SOX9 binding to the element, but it did not alter the weak recognition by Hmgb1 (compare Figs. 5B, 12A and D). Hmgb1 somewhat increased SOX9 binding to Pe1M1, while it decreased L-Sox5 binding.

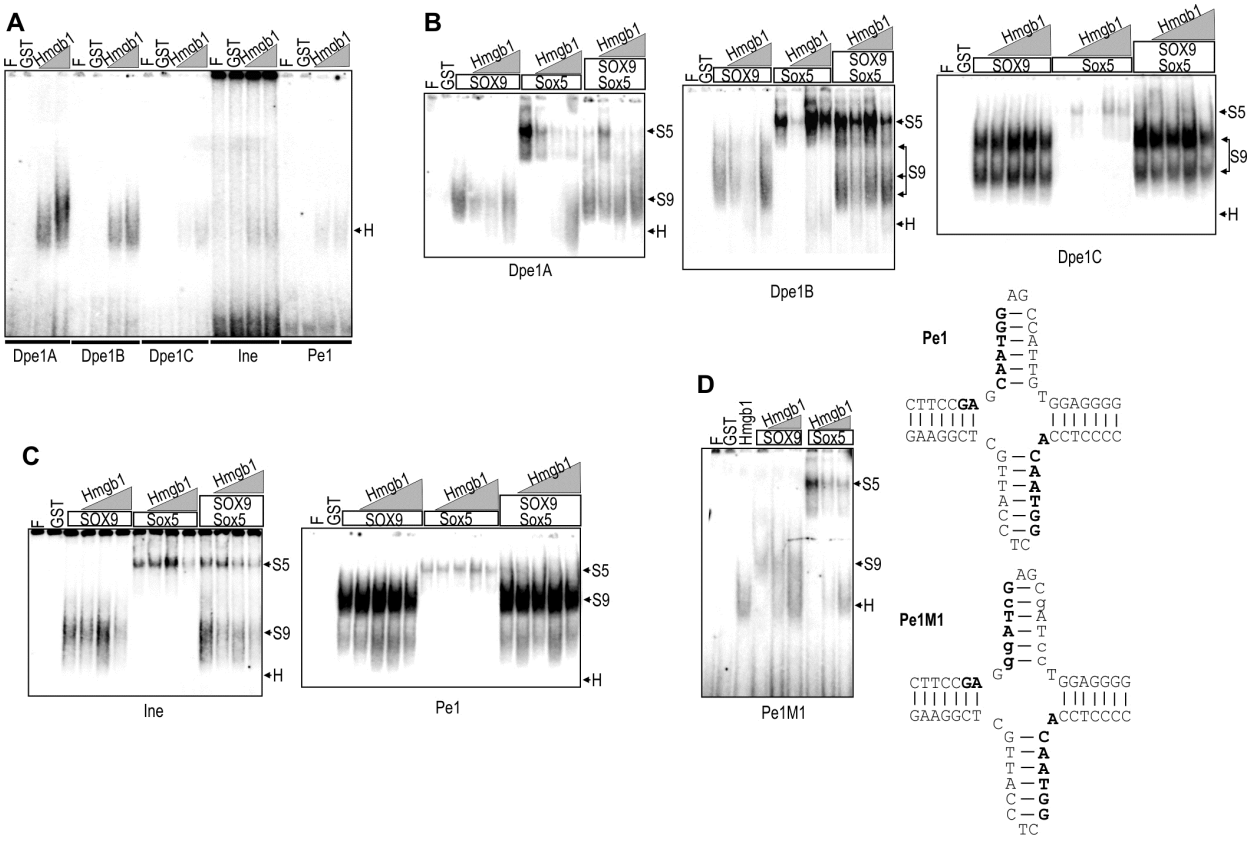

Fig. 12. Hmgb1 binds to conserved *Matn1* control elements *in vitro*. (A) Purified GST-fused Hmgb1 binds the DNA elements with variable efficiency in EMSA. Binding of purified SOX9 and L-Sox5 to Dpe1 (B) and short promoter elements Ine and Pe1 (C) in the presence of increasing amount of purified Hmgb1. (D) Hmgb1 binds with similar efficiency to Pe1 and Pe1M1 harboring palindrome sequences. F, free probe; H, Hmgb1 complex; S5, L-Sox5 complex; S9, SOX9 complex.

These data show that Hmgb1 can interact with *Matn1* control elements Dpe1, Pe1 and Ine *in vitro* and it can modify the binding of Sox proteins.

5.13. Compensatory effect of Hmgb1 on proximal promoter mutations

Next we tested how point mutations in the Sox or other sites of Pe1 and Ine [88] affected the Hmgb1-facilitated activation of the *Matn1* long promoter by the Sox trio (Fig. 13). As opposed to the lack of any significant effect in CEC culture (Fig. 13C), Hmgb1 increased the Sox trio-mediated synergistic activation of the wild-type promoter and some of its mutants in LDM culture (Fig. 13B).

Thus, it doubled the activity of derivatives ΔPe1M1 or ΔIneM1, carrying mutations in the Sox site of Pe1 or the 5' Sox site of Ine, respectively. It also elevated the activity of ΔPe1M4 lacking factor binding to the Sox spacer. In contrast, mutation IneM2, disrupting both Sox sites and an unknown factor binding site of Ine, obstructed the positive effect of Hmgb1, decreasing

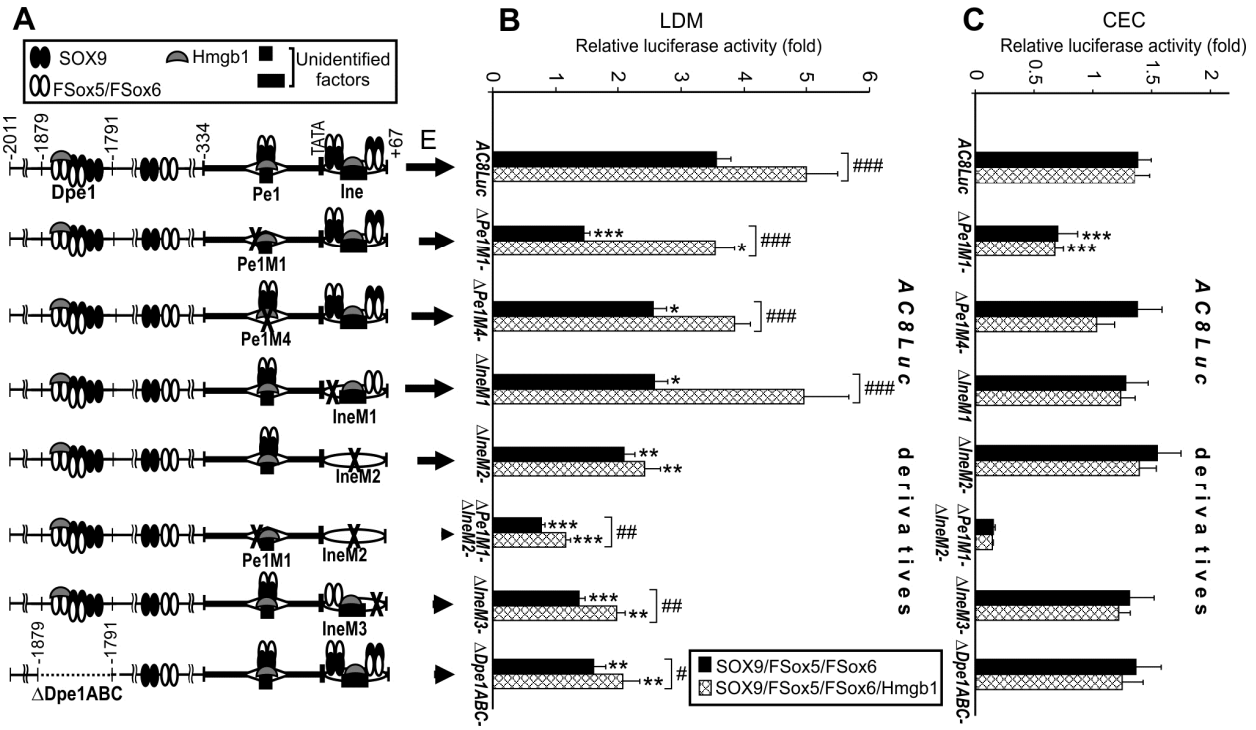

Fig. 13. Effect of Hmgb1 on the Sox trio-mediated transactivation of the long promoter and its mutant derivatives. (A) The schematic (not drawn to scale) illustrates factor binding to the short promoter and upstream elements. Arrows indicate transcriptional activities at early stage of chondrogenesis (E). Sox proteins were coexpressed with and without Hmgb1 in LDM (B) and CEC cultures (C) and reporter activities were expressed in fold values relative to that for *AC8Luc* cotransfected with vectors. *p<0.05, **p<0.01, ***p<0.001 mutants vs. similarly cotransfected *AC8Luc*; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 as indicated.

the activity to half. In spite of the significant, but small activation by Hmgb1, deletion of Dpe1ABC or mutation in the 3' Sox site of Ine (IneM3) or the double mutant Pe1M1/IneM2 decreased the transactivation by 60-76%.

Thus in early stage of chondrogenesis, Hmgb1 can partly or fully compensate for the lack of factor binding to the Sox site of Pe1 and its spacer or to the 5' Sox site of Ine. However, it could not compensate for the lack of factor binding to the 3' or both Sox sites of Ine or to Dpe1. Pe1M1, Pe1M4, and IneM1 did not destroy the palindrome structure of Pe1 and Ine, whereas IneM3 and IneM2 disrupted one or both palindromes of Ine, respectively (Fig. 5A) [88, 89]. Dpe1 also harbors palindrome sequences (Fig. 5). This suggests that Hmgb1 facilitated the Sox trio-mediated activation of the promoter and some promoter mutants not disrupting the palindrome structures of the elements. One explanation can be that Hmgb1 may bind to such palindrome sequences and by prebending the DNA, it may facilitate the binding of Sox and partner factors to the *Matn1* control elements in early chondrogenesis, thereby compensating for the negative effect of point mutations.

5.14. Effect of Hmgb1 silencing on the expression of cartilage-specific genes

To test the effect of Hmgb1 on the endogenous *Matn1* expression in later steps of chondrogenesis, we performed silencing experiments in chondrogenic cell lines. The established human cell lines of either costal chondrocyte (C-28/I2) or chondrosarcoma origin (SW1353) are known to express cartilage-specific genes at a relatively low level compared to primary cultures [90, 100], whereas the chondrogenic marker gene expression of the RCS cell line is more similar to that of primary cultures [91]. In consistency with these data, we measured low mRNA levels relative to that of the S18 ribosomal protein mRNA in the human cell lines for *MATN1* ($3.99x10^{-5}$ in C-28/I2 and $9.74x10^{-5}$ in SW1353 cells) and *COL2A1* ($4x10^{-5}$ in C-28/I2 and $1x10^{-5}$ in SW1353 cells) (Fig. 14A and B). *SOX9* expression level was also relatively low ($7.16x10^{-2}$ in C-28/I2 and $4.74x10^{-2}$ in SW1353 cells), in sharp contrast to the highly elevated *HMGB1* mRNA expression of the cells (1.59 in C-28/I2 and 1.46 in SW1353). RCS cells exhibited much higher relative mRNA levels for *Col2a1* ($8x10^{-2}$), *Sox9* (2.51) and *Matn1* ($3.56x10^{-4}$), whereas the *Hmgb1* mRNA expression ($1.39x10^{-1}$) was somewhat lower in the rat than in the human cell lines (Fig. 14C).

As these cells lines differed from primary cultures in their high HMGB1 and low chondrogenic marker gene expressions [90, 91, 100], they provided excellent tools to test the hypothesis in silencing experiments whether the highly elevated *HMGB1* expression as compared to *Sox9* may contribute to the repression of the chondrogenic marker genes in later stage of chondrogenesis. We found that silencing of *HMGB1* by 2.5-fold in C-28/I2 cells increased the expression level for *MATN1* and *COL2A1* by 3.6-4.3-fold and 4.9-5.3-fold, respectively (Fig. 14A). Silencing of *HMGB1* by 2.4-2.7-fold in SW1353 cells increased the expression level for *MATN1* and *COL2A1* by 9.6-32-fold and 10-13-fold, respectively (Fig. 14B). The activation was specific, as it affected neither the *GAPDH* nor the *SOX9* mRNA levels (Fig. 12A and B). When silencing decreased the relative *Hmgb1* mRNA level of RCS cells by 42-57-fold below the level of *Sox9*, the *Matn1* and the *Col2a1* mRNA levels increased by 59-74-fold and 9.3-15.9-fold, respectively (Fig. 14C). *Hmgb1* silencing however did not significantly alter the *Hprt* or the *Sox9* levels, suggesting a specific effect.

We concluded that the highly elevated *Hmgb1* expression compared to *Sox9* can inhibit the cartilage protein gene expression in the established chondrogenic cell lines. In consistency with the repression of the *Matn1* promoter activity by high dose of Hmgb1 in CEC culture (Fig. 11A), these data support the hypothesis that large abundance of Hmgb1/HMGB1 may interfere with the Sox trio-mediated activation of *Matn1, Col2a1* and their human orthologs. This suggests that

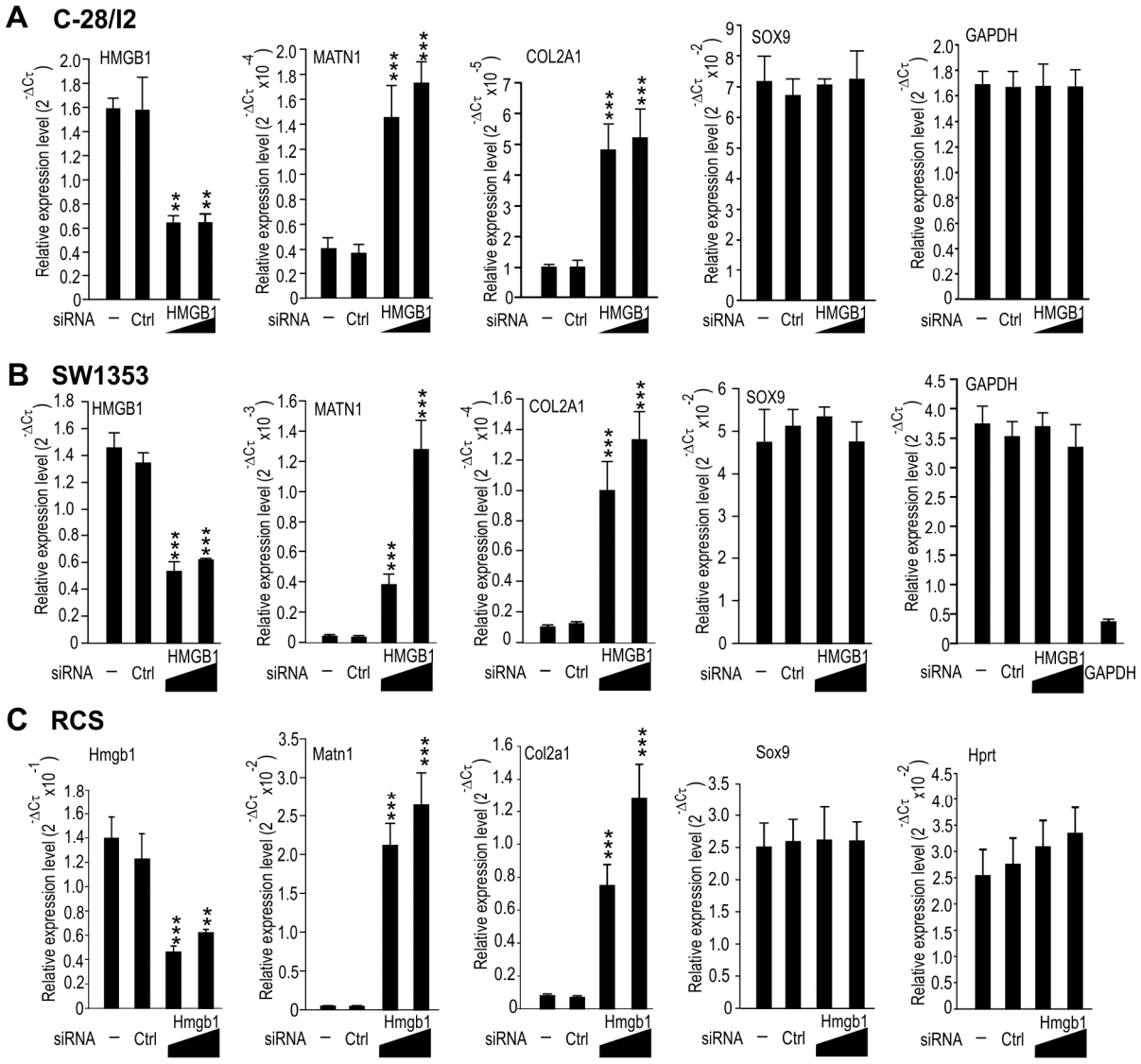

Fig. 14. Effect of *Hmgb1* silencing on chondrogenic marker gene expression. (A- B) Established human chondrogenic cell lines were transfected with increasing amount of HMGB1 siRNA (100 pmoles and 200 pmoles for C-28/I2; 200 pmoles and 400 pmoles for SW1353). Parallel plates transfected with control siRNA (Ctrl) and GAPDH siRNA served as negative and positive controls, respectively, to indicate the efficiency and specificity of silencing. (C) Similar transfection experiments were performed in the RCS cell line using increasing amount (200 pmoles and 400 pmoles) of rat Hmgb1 siRNA and negative control siRNA. (A-C) Marker gene expression levels were determined by QRT-PCR and plotted relative to the invariant RPS18/rps18 mRNA levels. *p<0.05, **p<0.01, ***p<0.001 vs. nontransfected samples.

Hmgb1 may inhibit the activation not only of Matn1 but of other cartilage protein genes as well by the Sox trio.

5.15. Model for the unique transcriptional regulatory mechanisms of the *Matn1*

Role of Sox trio and Nfi in the regulatory mechanism of *Matn1*

Finally, we proposed a model for the unique transcriptional regulatory mechanisms of *Matn1*. This hypothesis is based on previous observations from our group [86, 88] that *in vivo* footprints were absent from the short promoter in the non expressing CEF and they gradually appeared in differentiating HDM culture, strongly suggesting that activation of *Matn1* involves regulation at chromatin level. In fact, the Nfi sites of SI and SII were not occupied *in vivo* in CEF, albeit Nfi genes are expressed in CEF [89] and Nfi proteins can bind SI and SII from CEF extracts in EMSA and *in vitro* footprinting [87]. Based on their interaction with histones [60, 72], Nfi proteins may help to disrupt the nucleosome structure during *Matn1* activation.

According to our model, the special geometric arrangement of proximal elements may explain the unique regulation of *Matn1*, as it allows fine tuning of the promoter activity by L-Sox5/Sox6 and Nfi, depending on their relative abundance to SOX9 (Fig. 15A). At the onset of chondrogenesis, binding of Sox and Nfi proteins might be needed to open the chromatin structure around the TATA (Fig. 15A, a and b). At early stage of chondrogenesis, when occupancy of the sites is low and SOX9 is expressed at high molar excess relative to L-Sox5/Sox6, SOX9 preferably binds Pe1, and the Ine-bound L-Sox5/Sox6 synergizes with SOX9 by likely increasing its efficiency to bind Pe1 (Fig.15A,b). Pe1 likely plays central role in enhanceosome formation and in the SOX9-mediated promoter activation from Dpe1 and other distal elements but, due to the low abundance of transcription factors, the transcription activity is low in early proliferative chondroblasts (Fig. 15A, b). The promoter activity is highest in late proliferative chondroblasts, where the occupancy of Pe1 and other elements by SOX9 and other factors is optimal (Fig. 15A, c). At late stage, however, when the Sox trio level is elevated, large molar excess of L-Sox5/Sox6 to SOX9 can decrease the transactivation by SOX9, possibly by competing with SOX9 for binding Pe1 and other elements (Fig. 15A, d). High occupancy of the Sox sites of Ine may even physically interfere with the recruitment of PIC to TATA. Overproduction of Nfi may also decrease the promoter activity due to competition between activator (e.g. Nfib) and repressor Nfi isoforms or by sterically blocking TBP binding to TATA (Fig. 15A, e).

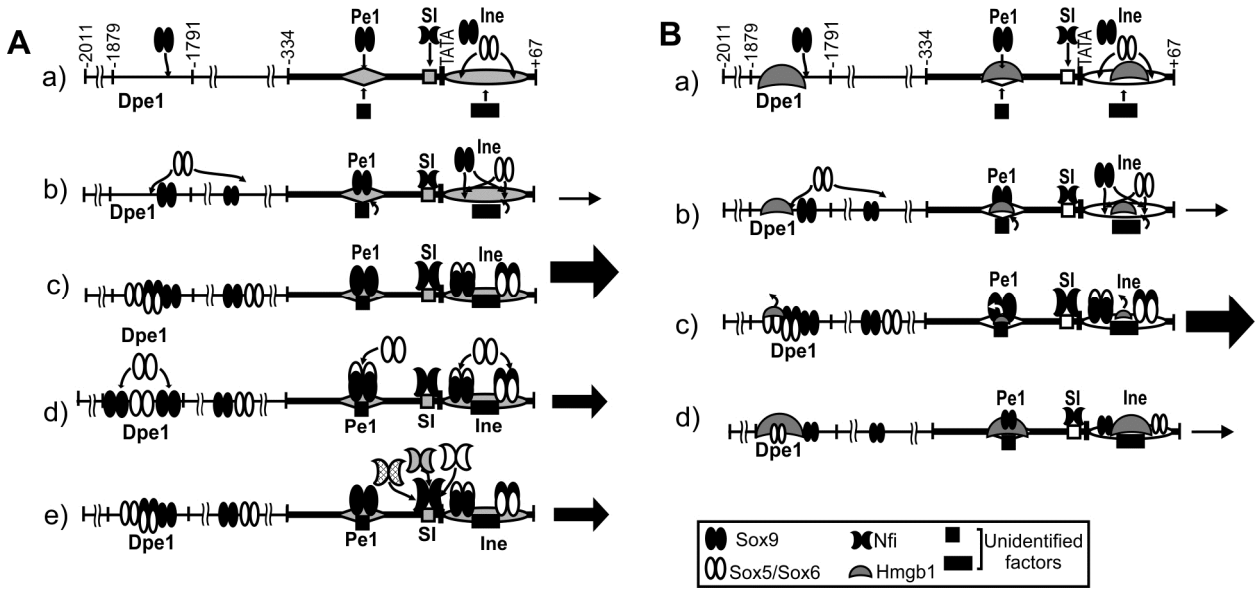

Fig. 15. Model for the role of Sox trio, Nfi and Hmgb1 factors in the unique transcriptional regulatory mechanisms of the *Matn1*. (A) Model for fine tuning of the promoter activity by the Sox trio and Nfi. Factor binding to DNA elements is schematized during *Matn1* activation at the onset of chondrogenesis (a); in early (b) and late proliferative chondroblasts (c) at low and optimal occupancy of sites, respectively; and in late stage at high occupancy of the Sox (d) or Nfi sites (e). (B) Model for the regulation of the *Matn1* promoter by Hmgb1 and Sox trio. Schematic illustration of factor binding to the conserved DNA elements at the onset of chondrogenesis (a), in early (b) and late proliferative chondroblasts (c) and in oncogene-transformed cell lines (d). See the text for detailed description.

Model for the interplay between Hmgb1 and Sox trio in the regulation of *Matn1*

Based on the data, we also suggest a model for the contribution of Hmgb1 to the activation of the gene at the onset of chondrogenesis in amniotes (Fig. 15B). Hmgb1 is bound to the Dpe1, Pe1 and Ine elements in fibroblasts and in committed mesenchyme (Fig. 15Ba). The architectural protein may fluidize the chromatin and bend the DNA to facilitate sequence-specific binding of the Sox trio to these DNA elements in early steps of chondrogenesis (Fig. 15Bb). The transcriptional activity of the gene is increasing as Hmgb1-binding is replaced by Sox9-binding and L-Sox5/Sox6 increases the transactivation by Sox9 in a dose-dependent synergistic manner in late proliferative chondroblasts (Fig. 15Bc). However, highly elevated Hmgb1 expression in transfected late proliferative chondroblasts or oncogenically transformed chondrogenic cells (e.g. chondrosarcomas), can repress the gene expression, likely because Hmgb1 present in large abundance may compete with Sox9- and Sox trio-binding to the conserved DNA elements (Fig. 15Bd).

6. DISCUSSION

By dissecting and characterizing the *Matn1* DNA elements and the interacting Sox, Nfi and Hmgb1 proteins, this dissertation gives new insight into the unique control mechanism that directs *Matn1* expression into specific chondroblast developmental stages and distinct growth plate zones. In agreement with former reports from our group [86-89], the results presented in this dissertation uncover 1) fine tuning of the Sox9-mediated synergistic activation of the *Matn1* promoter by the dose of L-Sox5/Sox6; 2) fine tuning of the Sox9-mediated *Matn1* promoter activation by the dose of Nfi proteins, which peaks in early stage of chondrogenesis; 3) the dose-dependent modulation of the Sox trio-mediated synergistic *Matn1* promoter activation by Hmgb1.

Furthermore, based on the present and former data of our group, we proposed a model for the unique transcriptional regulation of *Matn1* (Fig. 15). According to our model, the unique arrangement of the conserved DNA elements around the TATA box is an important part of this control mechanism, in which the Pe1-bound Sox9 plays a key role and mediates promoter enhancement from Dpe1. Chondroblast stage-specific regulation is achieved, because the Pe1-bound Sox9 activity is finely tuned by the doses of L-Sox5/Sox6 and Nfi proteins bound to Ine and SI, respectively. The promoter activity is highest in late proliferative chondroblasts, where - due to the high expression level of chondrogenic Sox genes - the occupancy of the elements is optimal and the Ine-bound L-Sox5/Sox6 synergistically increases the transactivation by the Pe1-bound Sox9, and the Sox9-mediated enhancement by Dpe1 is also optimal. In early stage of chondrogenesis, however, when the declining Hmgb1 level is higher than the raising Sox9 (Sox trio) level, Hmgb1 bound to the conserved *Matn1* DNA elements may fluidize the chromatin, thereby facilitating the binding of Sox9 (Sox trio) to the promoter elements and helping the activation of the gene. Nfi proteins expressed in early proliferative chondroblasts may also promote the disruption of the nucleosome structure, participate in the assembly of the PIC and contribute to promoter activation. In hypertrophic chondrocytes or under disease conditions (e.g. inflammation, tumorigenesis), the highly elevated Hmgb1 level may inhibit the promoter activity by competing with Sox proteins for their binding to the conserved *Matn1* control elements.

Results presented in this dissertation confirm SOX9 binding to the Pe1 and Ine elements observed in previous *in vitro* assays [86, 88, 89] and support the important role of these elements in the cartilage-specific promoter activity in line with former observations in genomic footprinting and transgenic mice [86, 88, 89]. The CEC-specific *in vivo* footprints detected at the paired Sox motifs of Ine and Pe1 in genomic footprinting are likely due to cartilage-specific

binding of Sox proteins [86, 89]. ChIP analysis performed by our collaborating partner at the University of Debrecen also demonstrated cell type-specific *in vivo* binding of the Sox trio to the *Matn1* control elements in human and rat chondrogenic cell lines. Furthermore, the presented mutational and functional data demonstrate the key role of the highly conserved Pe1 in the tansactivation by SOX9 and in SOX9-mediated enhancement from Dpe1 and distal elements. Ine is less conserved, but it is also needed for high transgene activity [88]. It rather seems to interact with L-Sox5/Sox6 in EMSA and forced-expression studies [88].

Our model can give an explanation why the *Matn1* short promoter plays a critical role in restricting cartilage-specific expression and how its activity is enhanced by distal elements in transgenic mice as it was observed earlier [86, 88]. Remarkably, the *Matn1* short promoter could even restrict the activity of a powerful Sox-driven pan-chondrocytic *Col2a1* heterologous enhancer to distal structures and specific growth plate zones. In line with our model, the transgene carrying the IneM1 mutation displayed very low activity in founder embryos, but this activity remained restricted to the columnar and prehypertophic growth plate zones, as with the TR70 transgene driven by the wild-type *Matn1* long promoter [88].

Multiple copies of the conserved Dpe1 element fused to the short *Matn1* promoter directed high, zonal and distal structure-specific transgene expression resembling to that directed by the long *Matn1* promoter [88]. We concluded that Dpe1 working as an important enhancer element may account in large part for the high Sox-mediated enhancement of the *Matn1* promoter in late proliferative chondroblasts for the following reasons. Dpe1 features three Sox sites binding SOX9 and L-Sox5 with opposite efficiency *in vitro*. It is needed for the high chondroblast stage-specific promoter activity and transactivation by the Sox trio. Dpe1 elements can exert a Sox trio-mediated, dose-dependent synergistic enhancement to the *Matn1* and *Col2a1* promoters in cultures.

Our model is strengthened by the remarkable sequence and positional conservation of proximal (short) and distal DNA element observed by our collaborating partner in Debrecen (Fig. 3) [86, 97] strongly suggesting an evolutionarily conserved transcriptional control in amniotes. Conservation of promoter and extragenic sequences in amniotes for other genes (e.g. *Sox2*) can reliably reflect their functional importance in development [40, 101]. Pe1 is most highly conserved among the *Matn1* control elements [86]. Sox sites of Pe1 recognized preferably by SOX9 95 bp to 195 bp upstream of TATA are most crucial for the promoter activity, but those of Ine preferably binding L-Sox5/Sox6 around the transcription start sites are also important. Sox proteins bind the two paired Sox site of Ine in a cooperative manner [88]. Dpe1 located 1800-2650 bp upstream of the promoter in various species is also highly conserved in amniotes [86].

As it was shown for Sox trio binding to other cartilage-specific control elements [52, 84], Sox proteins may also bind the three paired Sox sites of Dpe1 in a cooperative manner. Cotransfection and forced expression assays and the analysis of various single and double mutants revealed that the SOX9-mediated activation of the long promoter as well as the 4XDpe1-*Col2a1* promoter construct is dose-dependently modulated by L-Sox5/Sox6 (Figs. 7).

Such a high degree of sequence and positional conservation among chicken and mammalian orthologs has not been found for other cartilage genes. Conserved cartilage-specific element has been identified only in the far upstream enhancer of the mammalian orthologs of *Acan*, but it is not conserved in amniotes [84]. Although cartilage-specific control elements with functional Sox sites were found in varying location, e.g. in the first intron, far upstream promoter, 5' untranslated or proximal promoter regions [52, 78, 84, 102-104], but they do not show similarity to the *Matn1* control region.

The transcriptional regulation of the *Matn1* involves similar as well as different molecular mechanism as compared to other cartilage protein genes. The Sox trio likewise plays essential roles in transcriptional regulation of other cartilage-specific genes [52, 84, 104], but *Matn1* is regulated differently by Sox trio than other cartilage ECM genes. One difference is that whereas Sox9 is sufficient for the activation of *Col2a1, Acan* and *Crtl1*, L-Sox5/Sox6 is also absolutely required for the activation of *Matn1*, as Matn1 mRNA was not detected in *Sox5*-/-;*Sox6*-/- mice [57]. Furthermore, we found that L-Sox5/Sox6 modulates the *Matn1* promoter activation by SOX9 in a dose-dependent manner. This effect is likely due to unique set of conserved DNA elements that are capable of interacting with Sox proteins with different efficiency.

In addition to the role of Sox trio, we also demonstrate the novel role of Nfi proteins in the gene regulation. We provide evidence that Nfi proteins also modulate the promoter activity and the SOX9-mediated transactivation in a dose-dependent manner. They increase the activation by SOX9 at low dose, but repress that at high dose. In agreement with the transient activation of Nfi genes during *in vitro* chondrogenesis in QRT-PCR, dominant negative mutation of Nfib interfered with chondrogenesis, while overexpression of the wild type Nfib increased the *Sox9* and *Col2a1* expression [69], Nfi sites mediating this regulation however have not been identified yet. By extending novel results and previous data from our laboratory (EMSA, *in vivo* footprinting) [87], we provide evidence that, in addition to the Sox trio, Nfi proteins binding SI near TATA also play critical role in fine tuning and enhancement of the chondroblast stage-specific activity of the *Matn1* promoter. Based on genomic footprinting, the Nfi motifs of SI and SII are first occupied by *in vivo* bound transcription factors (Fig. 4), in line with the transient Nfi expression in early chondrogenesis [89].

Our model is consistent with former observations [86, 88] that *in vivo* footprints were absent from the *Matn1* short promoter in the non-expressing CEF and they gradually appeared in differentiating HDM culture, strongly suggesting that activation of *Matn1* involves regulation at chromatin level. In fact, the Nfi sites of SI and SII were not occupied *in vivo* in CEF, albeit Nfi genes are expressed in CEF (Fig. 4D) [89] and Nfi proteins can bind SI and SII from CEF extracts in EMSA and in genomic footprinting [87]. Based on their interaction with histones and GTFs [60, 72], we can assume that Nfi proteins may help to disrupt the nucleosome structure and contribute to *Matn1* activation.

Furthermore, we reveal a previously unknown role for Hmgb1 in cartilage-specific gene regulation and in tumorigenesis. *Hmgb1*$^{-/-}$ mice were previously shown to suffer from pleiotropic defects. They die from hypoglycemia after birth, but survivors rescued by glucose treatment show severe developmental retardation and abnormalities in skeletal development [105]. Detailed analysis revealed a delay in endochondral ossification, largely because the lack of Hmgb1 secretion by hypertrophic chondrocytes impaired cartilage invasion by blood vessels, osteoclasts, and osteoblasts [8]. The size of the developing cartilaginous elements stained by alcian blue was also smaller in E16.5 *Hmgb1*$^{-/-}$ embryos than in wild-type littermates [8], suggesting that early steps of endochondral bone formation may also be affected by the mutation. Furthermore, Hmgb1 and Hmgb2 are needed for posterior digit development [8]. In line with in situ hybridizations by others [8, 48], we found that *Hmgb1* expression declined during *in vivo* and *in vitro* chondrogenesis in inverse correlation with the activation of chondrogenic Sox and ECM genes, showing some overlap with raising *Sox9* expression just before and at the time of *Matn1* onset in early chondroblasts. Hmgb1 and SOX9 recognized the Dpe1, Pe1 and Ine elements with a reciprocal binding efficiency *in vitro*. In forced expression assays, optimal dose of Hmgb1 augmented the Sox trio-mediated activation of the *Matn1* promoter in early chondrogenesis as well as the ectopic induction of the endogenous *Matn1* in COS-7 cells by 2-3-fold. Similar effect of Hmgb1 transfection was reported in other systems [45]. Hmgb1 could in large part compensate for the effect of mutations in Pe1 and in the 5' Sox site of Ine in early chondrogenesis. In agreement with the slow occupancy of Sox motifs of the Pe1 and Ine elements of *Matn1* in genomic footprinting in HDM cultures undergoing chondrogenesis in vitro [86, 89] ChIP experiments performed by our collaborating partner at the University of Debrecen revealed specific, but weak Sox9 and Sox5/Sox6 binding to the conserved Dpe1, Pe1 and Ine elements of *Matn1* in rat and human chondrogenic cell lines. Hmgb1, however, exhibited much stronger binding to the same elements in agreement with the high *Hmgb1* but low *Matn1* and *Col2a1* expression of these cells. Elevated Hmgb1 level may be due to the oncogenic

transformation of these cells, since Hmgb1 is upregulated in cancer cells and plays a role in tumor growth [47]. Silencing of *HMGB1* specifically and markedly increased the expression of cartilage protein genes but not that of *SOX9*. When silencing decreased the *Hmgb1* mRNA level below 2% of that of *Sox9* in RCS cells, the *Matn1* and *Col2a1* expression increased even more dramatically, indicating that large abundance of Hmgb1 can inhibit the Sox9-mediated activation of cartilage protein genes. Hmgb1 overexpression in CEC also inhibited the *Matn1* promoter activity, indicating a conserved mechanism in amniotes. Our data strongly suggests that, by modulating the access of Sox factors to evolutionarily conserved DNA elements, Hmgb1 can facilitate the synergistic activation of *Matn1* by the Sox trio in early chondrogenesis, which turns to inhibition later. Thus Hmgb1 expressed in large abundance in terminally differentiated chondrocytes may compete with Sox factors for binding to the elements, thereby contributing to the decreased *Matn1* expression during hypertrophy. Hmgb1 may also regulate other cartilage ECM genes by similarly modulating Sox factor binding to their control elements. Interestingly, HMGB1 was also reported to work as an alarmin and its level is high in the joints of patients with rheumatoid arthritis and juvenile idiopathic arthritis [106-108]. Therefore, based on our silencing experiments, we can assume that HMGB1 may similary inhibit the activity of *MATN1* and other cartilage ECM genes in such patients.

The unique molecular mechanism described in this dissertation may facilitate the construction of growth plate zone-specific vectors and the development of biotechnological therapies for skeletal diseases.

7. SUMMARY OF NOVEL RESULTS OF THE THESIS

1) We monitored the marker gene expression by QRT-PCR in cultures representing subsequent stages of chondrogenesis. We observed an inverse correlation between the raising Sox9 (and L-Sox5) and the declining Hmgb1 mRNA levels, showing only a limited overlap at the time of *Matn1* activation in early proliferative chondroblasts (at day 4 HDM culture). Transient activation of Nfi genes peaked in early proliferative chondroblasts (at day 4 HDM culture).

Dose-dependent promoter activation by the Sox trio:

2) We demonstrated in comparative EMSA experiment, that purified, GST-tagged Sox proteins can bind the conserved *Matn1* DNA elements with varying affinity. SOX9 binds most strongly to Pe1, followed by the 3' part of Dpe1, whereas L-Sox5 recognizes most efficiently the 5' part of Dpe1. Ine exhibited the weakest binding to the Sox trio.
3) Analysis of mutants in transient expression assays confirmed the important role of the Sox-binding sites of Pe1 and Ine in the high chondroblast stage-specific activity of the long promoter and its transactivation by SOX9 and the Sox trio at both early and later stages of chondrogenesis. Point mutation of the Pe1 Sox motif dropped, while double mutation (Pe1M1-IneM2) disrupting each of the three paired Sox motifs of Pe1 and Ine, completely diminished the transactivation of the long promoter by the Sox trio in forced expression studies in COS-7 cells. Pe1M1-IneM2 double mutation also interfered with the short promoter activation by the heterologous *Col2a1* cartilage-specific enhancer elements.
4) We demonstrated that Dpe1 also plays an important role in the chondoblast stage-specific promoter activation. Deletion of Dpe1 robustly decreased the long promoter activity in transient expression assays and it highly interfered with the promoter activation by SOX9 and the Sox trio in cotransfection and forced expression assays, especially at early stage of chondrogensis.
5) We confirmed in cotransfection experiments that Dpe1 can function as a cartilage-specific enhancer element in the non-expressing COS-7 culture, because four copies of Dpe1 worked efficiently in both orientations and the Dpe1 elements exerted a Sox trio-mediated dose-dependent synergistic enhancement to the homologous and the heterologous *Col2a1* promoters.
6) We provided evidence that, differing from other cartilage ECM genes, L-Sox5/Sox6 increased the transactivation of the *Matn1* long promoter by SOX9 in a dose-dependent synergistic

manner. At low dose (early stage) L-Sox5/Sox6 synergized with SOX9 to activate the promoter, while at high dose (late stage) L-Sox5/Sox6 inhibited the activation by SOX9.

Dose-dependent promoter activation by Nfi proteins:

7) We demonstated in transient expression and forced expression experiments that the Nfi-binding site of the SI element is also essential for the *Matn1* promoter activity and its activation by SOX9. Point mutations in the Nfi motifs of the SI element dropped the long promoter activity even in early proliferative chondroblasts and hampered the promoter activation by SOX9 in cotransfection and forced expression assays. Mutation of the SI element also decreased the short promoter activation by the heterologous *Col2a1* cartilage-specific enhancer elements.
8) We provided evidence in cotransfection and forced expression experiments that Nfi proteins can modulate the SOX9-mediated transactivation of *Matn1* long promoter in a dose-dependent manner. They increase the activation by SOX9 at low dose, while they decrease it at high dose.

Dose-dependent modulation of the promoter activity by Hmgb1:

9) In agreement with the QRT-PCR data, we found that Hmgb1 immunofluorescence dropped in the developing limbs of mouse embryos as chondrogenesis progressed, exhibiting a complementary pattern to that of Sox9 and Matn1 in overtly differentiated cartilaginous elements. Hmgb1 and Sox9 expression overlapped only in early chondroblasts at the time of the onset of *Matn1*. These data suggested that Hmgb1 may also contribute to the *Matn1* regulation in early steps of chondrogenesis.
10) We demonstrated that Hmgb1 can bind to the conserved *Matn1* DNA elements *in vitro* with opposite binding efficiency than SOX9.
11) We found that Hmgb1 can increase the *Matn1* promoter activity in cotransfection assays in early stages of chondrogenesis, but it rather inhibits the promoter activity at late stage.
12) We provided evidence that Hmgb1 can increase the transactivation of the *Matn1* promoter and even induce the endogenous *Matn1* by forced expression of the Sox trio in the non-expressing COS-7 cells.
13) We concluded that the high HMGB1/Hmgb1 level relative to that of SOX9/Sox9 may inhibit the SOX9/Sox9 mediated activation of *MATN1/Matn1* and other cartilage ECM genes in oncogene-transformed cell lines, because silencing of *HMGB1*/*Hmgb1* in established human

(C-28/I2, SW1353) and mouse chondrogenic cell lines (RCS) robustly increased the *MATN1/Matn1* and *COL2A1/Col2a1* expression levels.

Model for the transcriptional regulation of *Matn1*

14) Finally, we proposed a model for the unique transcriptional regulatory mechanisms of *Matn1*. According to this model chondroblast stage-specific activation of *Matn1* is achieved via a complicated interplay between Sox and Nfi proteins and the uniquely arranged conserved DNA elements. As a result, the transactivation of the *Matn1* promoter by Sox9 is fine-tuned by the varying doses of L-Sox5/Sox6 and Nfi proteins relative to Sox9 during chondrogenesis. Furthermore, Hmgb1 can facilitate the promoter activation by the Sox trio at early stage of chondrogenesis by loosening up the nucleosome structure and promoting Sox factor binding. However at late stage or in tumors, the highly elevated Hmgb1 abundance relative to Sox9 may inhibit the *Matn1* promoter activity by competing with the Sox trio for binding to the conserved DNA elements.

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9. SUPPLEMENTARY

Table S1. List of *Gallus gallus* primers used in QRT-PCR reactions and marker gene expression levels in cell cultures

Gene symbol	Definition	Acc. No.	Forward sequence	Reverse sequence	Relative expression level ($2^{-\Delta Ct}$)*			
					CEF	HDM day 0	HDM day 4	CEC
Matn1	matrilin-1, cartilage matrix protein	NM_001030375	tccacagggaccatgacc	ctggccccttctgtgtca	3.4E-07	4.2E-06	9.3E-04	8.7E-03
Matn3	matrilin 3	NM_205072	ctcagaagctgcaagcctatc	gtgagtagaacgctggctca	8.9E-07	6.8E-06	8.1E-06	1.6E-04
Matn4	matrilin 4	XM_425698	gacctggtgatggtgatcg	caccagctcgaagttctgg	3.2E-06	2.4E-04	2.0E-03	1.6E-05
Col2A1	collagen, type II, alpha 1	NM_204426	tgccgcgacatcaaactct	ctggttcgggtcaatccagta	2.7E-03	2.3E-03	1.8E-02	4.1E-01
Col6A1	collagen, type VI, alpha 1	NM_205107	gacatcatgctgttggtgga	ggtggtgtcaaagttcttgct	1.2E-03	5.4E-04	4.9E-03	8.5E-04
Col11A1	collagen, type XI, alpha 1, variant 2	XM_001231650	atccaatgggcactcagaac	ggtggcagagctgcaagt	7.5E-04	8.9E-04	1.1E-02	5.0E-02
Sox9	SRY (sex determining region Y)-box 9	NM_204281	catcgatttccgagacgtg	tttcgatgttggagatgacg	2.3E-05	2.6E-04	6.6E-04	4.3E-03
Sox6	SRY (sex determining region Y)-box 6	XM_421000	tgatcagcttacgggagca	aggctgccagctttttctg	3.3E-05	1.4E-04	2.3E-04	3.7E-04
Sox5	transcription factor L-Sox5-I	AJ626989	aacagccctcctcccaaaag	ttgggtttggctgaaaggtt	1.5E-04	2.1E-04	2.0E-04	3.1E-03
Hmgb1	high-mobility group box 1	NM_204902	gatccaaatgcaccgaaga	tggacgaaactcagagcaaa	6.5E-03	4.5E-03	1.5E-03	2.0E-04
Hmgn1	high-mobility group nucleosome binding domain 1	NM_205106	catcagtagaattgcttatctcgtg	cattactcgtcaagaaatccaaca	4.7E-05	2.4E-04	1.5E-04	6.1E-05
Nfia	nuclear factor I-A	NM_205273	tgatcagcttacgggagca	caacagcgagttggtaagca	1.2E-03	9.2E-04	5.2E-03	9.0E-04
Nfib	nuclear factor I-B	NM_205272	ctggtataaatctgcagaggtcttt	gagatagttttgggtcttttgctg	5.9E-04	3.1E-04	8.2E-04	5.6E-05
Nfic	nuclear factor I-C	NM_205271	gctctgcaattagccactttcc	catcctgggtcaggcagaga	7.7E-04	7.5E-04	4.4E-03	3.4E-04
Nfix	nuclear factor I-X	NM_205270	catcagtagaattgcttatctcgtg	cattactcgtcaagaaatccaaca	1.0E-05	2.6E-06	5.8E-05	3.3E-06
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	NM_204305	cctctctggcaaagtccaag	accatgtagttcagatcgatgaag				
28S rRNA	28S ribosomal RNA	DQ018756	cccagaaaaggtgttggttg	cttagcgggctccgactt				
18S rRNA	18S ribosomal RNA	AF173612	gcaattattccccatgaacg	atcaacgcgagcttatgacc				

*Gene expression levels are given relative to the three internal control genes.

Table S2. List of primers used in QRT-PCR reactions in forced expression and silencing experiments

Gene symbol	Definition	Acc. No.	Forward sequence	Reverse sequence
hMATN1	matrilin-1, cartilage matrix protein	NM_002379	gaacagcgacggcaagac	atgaggaagaccaggtcagtg
hCOL2A1	collagen, type II, alpha 1	NM_033150	ccctggtcttggtggaaa	cattggtccttgcattactcc
hHMGB1	high-mobility group box 1	NM_002128	cattgagctccatagagacagc	ggatctcctttgcccatgt
hSOX9	SRY (sex determining region Y)-box 9	NM_000346.3	gtacccgcacttgcacaac	tctcgctctcgttcagaagtc
hGAPDH	glyceraldehyde-3-phosphate dehydrogenase	NM_204305	cctctctggcaaagtccaag	accatgtagttcagatcgatgaag
hRPS18	ribosomal protein S18	NM_022551	cttccacaggaggcctacac	cgcaaaatatgctggaacttt
rMatn1	matrilin-1, cartilage matrix protein	NM_001006979	ttggcaagaagttgcagaaa	cactatggactcacaggcaca
rCol2A1	collagen, type II, alpha 1	NM_012929	ctggtacccctggaaatcct	ccatctgggctgcaaagt
rHmgb1	high-mobility group box 1	XM_003751117	gtaattttccgcgcttttgt	tcatccaggactcatgttcagt
rSox9	SRY (sex determining region Y)-box 9	XM_003750950.1	gtacccgcatctgcacaac	ctcctccacgaagggtctct
rHprt	hypoxanthine-guanine phosphoribosyl transferase	NM_012583	gaccggttctgtcatgtcg	acctggttcatcatcactaatcac
rRps18	ribosomal protein S18	NM_213557	cttccacaggaggcctacac	cggggatcactagggacat
mGapdh	glyceraldehyde-3-phosphate dehydrogenase	NM_001195426	gctcgtcatcaatggaagc	ttgattttggagggatctcg

h: Homo sapiens r: Rattus norvegicus m: Macaca mu