Characterization of the hypertrophic chondrocyte-specific Col10a1 cis-enhancer both in vitro and in vivo

Li, F; Lu, Y; Abbassi, S; Ding, M; Chen, Y; Wang, S; Lee, B; *Zheng, Q
*Rush University Medical Center, Chicago, IL; Baylor College of Medicine, Houston, TX; Anhui Medical University, Hefei, China
Qiping_Zheng@rush.edu

INTRODUCTION:
The type X collagen gene (Col10a1) is a specific molecular marker of hypertrophic chondrocytes, a cell stage that is critical for chondrocyte terminal differentiation, linking both cartilage and bone formation during embryonic and postnatal skeletal development. Mutations in human COL10A1 cause Schmid Metaphyseal Chondrodysplasia. Abnormal COL10A1 expression and altered chondrocyte maturation have been associated with a spectrum of human skeletal disorders as well as joint degeneration as seen in osteoarthritis (OA). Therefore, characterization of the molecular regulation of hypertrophic chondrocyte-specific Col10a1 expression is essential to understand the molecular processes that specify endochondral bone formation and the molecular pathogenesis of skeletal dysplasias and osteoarthritis.

However, until recently, the molecular mechanisms that regulate cell-specific Col10a1 expression, i.e. the refined cis-enhancer element and its putative binding factors that together specify Col10a1 expression during chondrocyte maturation remain unidentified. We have recently shown that a 90-bp deletion mutant cis-enhancer element (combined –4296 to -4280 bp and –4240 to –4171 bp) within the 150-bp Col10a1 distal promoter (–4296 to –4147 bp) is sufficient to direct its hypertrophic chondrocyte-specific expression in transgenic studies (Zheng et al., 2003 and 2009). Interestingly, detailed sequence analysis of this region identified two tandem repeat putative Runx2 binding sites (TGTGGGTGTGGC, –4187 to –4176) compared to the Runx2 binding site [TGTGGC] that we have previously described (Zheng et al., 2003). We therefore hypothesize that Runx2 may interact with this short enhancer via the tandem repeat Runx2 binding sites and regulate cell-specific Col10a1 expression.

METHODS:
Generation of transgenic reporter construct:

The Tg-6x5083 transgenic reporter construct was generated by inserting six copies of the 5-prime sequence (–4296 to –4214 bp) of the 150-bp cis-enhancer upstream of the Col10a1 basal promoter (–220 to +110 bp) to drive LacZ as a reporter. Additional reporter constructs using elements derived from the 150-bp Col10a1 distal promoter (–4296 to –4147 bp) were described in following sections.

Electrophoretic mobility shift assay:

A series of six long (~50-bp) and eleven short (~30-bp) overlapping pairs of DNA oligos derived from the 150-bp Col10a1 distal promoter have been commercially synthesized by IDT Technologies. Mutations in or outside of the putative Runx2 core binding sites were also used for the Electrophoretic mobility shift assays. EMSA assay using MCT cell nuclear extracts as well as these annealed DNA oligos as probes have been carried out as previously described (Zheng et al., 2003).

Cell culture and transfection studies:

MCT cells were grown at 32°C in standard DME media with 8% FBS (GIBCO BRL) and 8% CO₂ as per published protocol. Reporter constructs were generated by inserting six copies of the series of cis elements covering the 150-bp cis-enhancer upstream of the Col10a1 basal promoter on a pcDNA3 backbone. The reporter plasmids as well as the pSAlgeoAP control one were transfected into hypertrophic MCT cells as previously described (Zheng et al., 2003).

Generation and histochemical analysis of transgenic mice:

The DNA fragment containing the entire transgenic cassettes was released by Not I and Sal I digestion. Purified DNA’s were redissolved by Not I and Sal I digestion. Purified DNA’s were redissolved by Not I and Sal I digestion. Purified DNA’s were redissolved by Not I and Sal I digestion. Purified DNA’s were redissolved by Not I and Sal I digestion. The reporter plasmids as well as the pSAlgeoAP control one were transfected into hypertrophic MCT cells as previously described (Zheng et al., 2003 and 2009).

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RESULTS:

Transgenic study using 5-prime sequence of the 150-bp promoter:

In order to refine the map of the major cis-element and to identify its putative binding factors that contribute to the regulation of Col10a1 expression, we further dissected this 150-bp Col10a1 distal promoter by generating additional transgenic mouse lines with reporter construct that uses the 5-prime sequence (~4296 to –4214 bp) to drive LacZ as a reporter. No cell-specific blue staining was observed in the hypertrophic chondrocytes of these transgenic mice (Figure 1).

EMSA Assay using cis-elements derived from the 150-bp promoter:

We have also performed Electrophoretic Mobility Shift Assay (EMSA) using a series of six long (~50-bp, probe-I to VI) and eleven short (~30-bp, probe-I to 11) overlapping pairs of DNA oligos and the hypertrophic MCT cell nuclear extracts. Specific DNA/protein complexes formed with the probe V and probe 9 which covers sequence from –4201 to –4163 bp and –4197 to –4171 bp respectively (Figure 2).

Moreover, no DNA/protein complexes formed when the putative core Runx2 binding sites were mutated, whereas mutations outside of the core binding sites do not abolish the binding complex (data not shown). Upregulated reporter activity was also observed in hypertrophic MCT cells by transfection studies using reporter constructs derived from these cis-elements (data not shown).

DISCUSSION:

Since no hypertrophic chondrocyte-specific expression (blue staining) was observed in the transgenic mice using the 5-primer sequence to drive LacZ gene. This result together with the transgenic study using the reporter construct of Tg-6x5083 suggests that the major cis-enhancer element for Col10a1 expression is within the 3-prime sequence from –4240 to –4171 bp. The in vitro DNA binding assay and the transfection studies that use cis-elements derived from the 150-bp promoter demonstrate that certain transcription factors bind this 30-40 bp short cis-element and mediate cell-specific Col10a1/reporter expression. Previously, Runx2 has been shown to regulate Col10a1 expression in different species by interacting with their promoters. Moreover, RUNX2 was recently shown to bind a putative RUNX2 binding site that includes TGTGGC as the core sequence (Agueda et al., 2008). Although under further investigation, our preliminary results suggest that Runx2 may interact with this short cis-enhancer via the tandem repeat Runx2 binding sites and regulate cell-specific Col10a1 expression.

REFERENCES:


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