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REVIEW

Col10a1 gene expression and chondrocyte hypertrophy during skeletal development and disease

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Abstract **The type X collagen gene, *COL10A1*, is specifically expressed by hypertrophic chondrocytes during endochondral ossification. Endochondral ossification is a well-coordinated process that involves a cartilage intermediate and leads to formation of most of the skeleton in vertebrates during skeletogenesis. Chondrocyte hypertrophy is a critical stage of endochondral ossification linking both bone and cartilage development. Given its specific association with chondrocyte hypertrophy, type X collagen plays essential roles in endochondral ossification. It was previously shown that transgenic mice with mutant type X collagen develop variable skeleton-hematopoietic abnormalities indicating defective endochondral ossification, while mutations and abnormal expression of human *COL10A1* cause abnormal chondrocyte hypertrophy that has been seen in many skeletal disorders, including skeletal chondrodysplasia and osteoarthritis. In this review, we summarized the skeletal chondrodysplasia with *COL10A1* gene mutation that shows growth plate defect. We also reviewed recent studies that correlate the type X collagen gene expression and chondrocyte hypertrophy with osteoarthritis. Due to its significant clinical relevance, the type X collagen gene regulation has been extensively studied over the past two decades. Here, we focus on recent progress characterizing the *cis*-enhancer elements and their binding factors that together confer hypertrophic chondrocyte-specific murine type X collagen gene (*Col10a1*) expression. Based on literature review and our own studies, we surmise that there are multiple factors that contribute to hypertrophic chondrocyte-specific *Col10a1* expression. These factors include both transactivators (such as Runx2, MEF2C etc.) and repressors (such as AP1, NFATc1, Sox9 etc.), while other co-factors or epigenetic control of *Col10a1* expression may not be excluded.**

Keywords *Col10a1* gene expression, *cis*-enhancer, transcription factors, AP1 and Runx2, chondrocyte hypertrophy, skeletal development and diseases

Introduction

The type X collagen gene (*Col10a1*) is specifically expressed when chondrocytes undergo hypertrophy or maturation during endochondral ossification (Warman et al., 1993). Endochondral ossification is a well-coordinated process and a major developmental pathway for most appendicular, as well as some of the axial skeleton. In this pathway, chondrocytes within the primordial cartilaginous model undergo proliferation, maturation, and hypertrophy and eventually replaced by bony tissue after the vasculature invasion and bone matrix deposition (Mackie et al., 2008). At an end stage of chondrocyte differentiation, hypertrophic chondrocytes play essential function during bone growth (Kronenberg, 2003) by associating with blood vessel invasion and calcified matrix for endochondral ossification (Linsenmayer et al., 1991). Type X collagen is an extracellular matrix protein that forms a homotrimer by three identical alpha1 chain (α1(X)) subunits, each containing a triple helical domain (COL) flanked by non-collagenous carboxyl- (NC1) or N-terminal (NC2) domains (Linsenmayeret al., 1985; Bateman et al., 2005). The exact function of type X collagen during chondrocyte

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maturation has not been fully elucidated. However, previous studies have clearly shown that type X collagen plays a role in normal distribution of matrix vesicles and proteoglycans within the growth plate. It has been shown that mice carrying a mutant *Col10a1* transgene develop skeletal deformities including compression of hypertrophic zone and decreased new bone formation (Jacenko et al., 1993). It has also been reported that *Col10a1* null mice show defect in mineralization and hematopoiesis and compressed growth plate, suggesting that type X collagen deficiency may impair the supporting properties of the growth plate and affect the mineralization process (Kwan et al., 1997). These observations indicate the requirement of type X collagen for normal skeletal morphogenesis. The type X collagen is needed for hematopoiesis and may facilitate endochondral ossification by providing a proper environment for mineralization and modeling. This is because type X collagen is not only the major component, it may also influence deposition of other matrix components to the hypertrophic zone of growth cartilage, where endochondral ossification occurs (Kwan et al., 1997; Shen, 2005; Grskovic et al., 2012).

Mutation and altered *COL10A1* expression in human skeletal dysplasias

Schmid metaphyseal chondrodysplasia (SMCD: MIM# 156500) is an autosomal dominant skeletal dysplasia that was first described to have a deletion mutation in human *COL10A1* (Warman et al., 1993). Later, multiple missense, nonsense, and frameshift mutations in *COL10A1* that cause SMCD were reported in many patients with multiple ethnic origins (Chan et al., 1995; McIntosh et al., 1995; Stratakis et al., 1996; Wallis et al., 1996; Ikegawa et al., 1997; Ikegawa et al., 1998; Sawai et al., 1998; Marks et al., 1999; Matsui et al., 2000; Gregory et al., 2000; Bateman et al., 2003; Bateman et al., 2004, 2005; Wilson et al., 2005; Mäkitie et al., 2005, 2010; Ho et al., 2007; Tan et al., 2008; Woelfle et al., 2011). These mutations either occur within a single codon and cause amino acid substitutions, or introduce premature stop codons and thus, result in truncated protein products (Gregory et al., 2000). Interestingly, a novel frameshift mutation (c2029delG) of *COL10A1* was recently reported in a Chinese family that will result in elongation of the deduced collagen alpha1 chain (α1(X)) product (Zhu et al., 2011). The typical clinical features of these SMCD patients are disproportionate short stature, coxa vara, and waddling gait (Warman et al., 1993). The disproportionate short stature is due to progressive shortening and bowing of the femora and tibiae, while shortening and sagging of the femoral necks cause *coxa vara* (Stratakis et al., 1996). The long bone abnormalities of thickened and irregular growth plates seen in SMCD are possibly due to ill-functioned type X collagen affecting chondrocyte hypertrophy during endochondral ossification, as *Col10a1* null mice also show compressed growth plate and other skeletal abnormalities similar to SMCD (Kwan et al., 1997).

Meanwhile, altered *COL10A1* expression and chondrocyte hypertrophy may be associated with other skeletal diseases. It was previously reported that haploinsufficiency of RUNX2, a master transcription factor for osteoblast differentiation and chondrocyte maturation, causes cleidocranial dysplasia, or CCD (Komori et al., 1997; Lee et al., 1997; Kim et al., 1999, Inada et al., 1999). CCD is characterized by delayed closure of the fontanel and hypoplastic clavicles that result from defective intramembranous ossification. Notably, characteristics of defective endochondral ossification were also observed in CCD patients (Lee et al., 1997). We have previously detected decreased *COL10A1* expression with markedly diminished hypertrophic zone in a fetal case of CCD (Zheng et al., 2005).

Regarding the potential mechanism of altered type X collagen expression causing skeletal disease, especially in SMCD, endoplasmic reticulum (ER) stress and an unfolded protein response (UPR) caused by intracellular retention of mutant protein (type X collagen etc.) has been suggested as an etiology of its occurrence, as generation of ER stress in hypertrophic chondrocytes induce cartilage-related pathological features of MCDS (Rajpar et al., 2009). Further analysis of the mutant type X collagen in transgenic mice confirmed that hypertrophic chondrocytes are able to cope with ER stress only to certain extent. If this capacity is surpassed, the UPR will be triggered and consequently, the features of cartilage pathology (hypertrophic zone expansion) occur (Kung et al., 2012).

COL10A1 expression and chondrocyte hypertrophy with osteoarthritis

Osteoarthritis (OA) is the most common joint disease. In recent years, type X collagen gene expression and chondrocyte hypertrophy have drawn extensive attention from scientists in the field of cartilage biology and OA research. This is because upon OA progression, the factors that constrain articular chondrocyte maturation are relieved and cells take on a mature phenotype that involves expression of hypertrophic hallmarks, including *Col10a1* (Pullig et al., 2000; Drissi et al., 2005). It has also been suggested that under OA conditions, articular chondrocytes undergo differentiation, hypertrophy and apoptosis which mimic the endochondral pathway (Kawaguchi, 2008). Correspondingly, the deposition of type X collagen has been observed at sites of OA lesions (Eerola et al., 1998), while upregulated *COL10A1* expression and enhanced chondrocyte hypertrophy were also observed in human osteoarthritic cartilage (von der Mark et al., 1992; von der Mark et al., 1995). There are also multiple lines of evidence which suggest a strong correlation of *COL10A1* expression and chondrocyte hypertrophy with OA. Runx2, a well-know transcription factor that controls both

Col10a1 expression and chondrocyte hypertrophy, was previously shown to be a candidate gene that promotes osteoarthritic progression (Kamekura et al., 2006). In surgically induced OA mouse models, Runx2 heterozygotes showed decreased cartilage destruction and osteophyte formation, along with reduced type X collagen and Mmp-13 expression. This suggested that Runx2 contributes to OA pathogenesis through chondrocyte hypertrophy (Kamekura et al., 2006; Kawaguchi, 2008). HIF-2α, which is encoded by *EPAS1*, is another potent *COL10A*1 transactivator as demonstrated by *COL10A1* promoter assay (Saito et al., 2010). Interestingly, Epas1-heterozygous mice showed resistance to OA development, while a human EPAS1 SNP (single nucleotide polymorphism) was shown to associate with knee OA in a Japanese population (Saito et al., 2010). Moreover, a most recent study has shown that a microRNA, named Hsa-miR-148a, was shown to interact with *COL10A1* and suppress its (as well as *MMP13* and *ADAMTS5* genes) expression and hypertrophic differentiation, while Hsa-miR-148a increases type II collagen and hyaline cartilage production (Vonk et al., 2014). Detailed information about chondrocyte hypertrophy and OA has recently been reviewed, which indicated that chondrocyte hypertrophy-like changes are observed in both human and experimental OA models, suggesting their roles in early and late stage OA (van der Kraan and van den Berg, 2012).

Col10a1 proximal regulatory elements

The above findings have clearly demonstrated that physiologic distribution of type X collagen during chondrocyte hypertrophy is essential for endochondral bone formation, whereas altered *Col10a1* expression is often accompanied by abnormal chondrocyte hypertrophy that is observed in multiple skeletal disorders. Understanding the cell-specific type X collagen expression in hypertrophic chondrocytes is essential for understanding the mechanism of skeletal development and may identify type X collagen gene regulators as therapeutic targets for relevant skeletal diseases. Previous studies on chicken chondrocytes have indicated that the regulation of type X collagen gene expression is at the transcriptional level (Beier et al., 1997). Therefore, for the past two decades, scientists have been focusing on characterization of the cis-regulatory elements and their binding factors that together mediate hypertrophic chondrocyte-specific type X collagen gene expression across species. Here, we focus on studies that have gradually and successfully characterized the type X collagen gene, especially murine *Col10a1 cis*-regulatory and enhancer elements. Comparative sequence analysis has previously demonstrated a high level of conservation among immediate upstream sequence of the transcription start site of human, murine, and chick *Col10a1* promoters (Beier et al., 1997). A 1.7 kb chick *Col10a1* promoter was able to direct tissue-specific reporter expression in hypertrophic chondrocytes (Jacenko et al., 1993). However, the corresponding mouse proximal promoter only direct non-specific reporter expression within the soft connective tissues, suggesting that the tissue-specific *Col10a1* cis-regulatory element is located within another region of the promoter (Eerola et al., 1998). The 120-bp murine *Col10a1* sequence (-120 to $+1$ bp) in the immediate vicinity of the start site of transcription was identified as likely the basal promoter (Beier et al., 1997). Meanwhile, *in vitro* transfection studies have identified multiple regulatory elements within human, murine, and chicken *Col10a1* promoter. These elements are within the proximal or distal promoter and can mediate both transactivating and repressive reporter activity to certain level (Long and Linsenmayer, 1995; Dourado and Lu Valle, 1998). About ten years ago, we reported identification of a 4.2-kb murine *Col10a1* regulatory element which includes both proximal and the basal promoter (-4018 to $+185$ bp). This element was only able to mediate reporter activity selectively in lower hypertrophic chondrocytes in transgenic mice, suggesting requirement of additional regulatory or enhancer elements for high-level tissue-specific murine *Col10a1*/reporter expression (Zheng et al., 2003).

Col10a1 cis-enhancer elements

In human *COL10A1*, both enhancer and silencer sequences were previously localized to its promoter regions by *in vitro* transfection studies using chondrogenic or non-chondrogenic cell lines (Beier et al., 1997). The enhancer element was further narrowed down to a 530-bp sequence (-2407 to -1870 bp) upstream of the transcription start site (Riemer et al., 2002). Meanwhile, a similar human *COL10A1* enhancer sequence was shown to confer both high-level and cell-specific type X collagen gene expression, although the reporter activity of this putative human enhancer was not tested *in vivo* (Chambers et al., 2002). Not surprisingly, this human *COL10A1* enhancer was later found to be highly conserved in the murine and bovine *Col10a1* genes. The murine enhancer element locating at the *Col10a1* distal promoter (-4.4 to -3.8 kb) shows 60-70% homology to the human enhancer and was able to drive high-level hypertrophic chondrocyte-specific reporter (*LacZ*) expression both *in vitro* and *in vivo* (Gebhard et al., 2004). The mammalian enhancer activity was further confirmed when a BAC (bacterial artificial chromosome) construct containing the complete *Col10a1* promoter was used to drive *LacZ* gene, as strong and robust *LacZ* activity was exclusively within the hypertrophic cartilage of the skeleton of the transgenic offspring (Gebhard et al., 2004). We have generated a number of transgenic (*Tg*) mouse lines using different (10-kb, 8-kb, 6-kb, and 4.6-kb) *Col10a1* promoter and intronic elements with or without the enhancer to drive *LacZ* gene (Zheng et al., 2009). The results demonstrated that transgenic constructs containing the 600-bp enhancer element (*Tg-10kb*, *Tg-6kb*,

and *Tg-4.6kb*) mediated high-level reporter activity, while the one without the enhancer (*Tg-8kb*) gives low-level reporter activity in hypertrophic chondrocytes (Zheng et al., 2009). To refine map the enhancer element, a series of reporter constructs were generated for transgenic studies. These constructs used multiple copies of the entire (600-bp) or partial (300-bp and 150-bp respectively) enhancer element and the *Col10a1* basal promoter (330-bp) to drive the *LacZ* gene. All these transgenic offspring show high-level hypertrophic chondrocyte-specific reporter expression, which narrows-down the *Col10a1* enhancer element to the 150-bp *Col10a1* distal promoter between −4296 and −4147 bp (Zheng et al., 2009).

Transcription factor AP1 and *Col10a1*

Activator protein-1 (AP-1) is a dimeric transcription factor formed by Jun proteins (c-Jun, JunB, JunD) and Fos proteins (c-fos, Fra-1, Fra-2, FosB, ΔFosB) (Eferl and Wagner, 2003; Eferl et al., 2004). Both Jun and Fos proteins have been shown to play essential roles during bone formation and cartilage development or chondrogenesis (Jochum et al., 2001; Hess et al., 2001; D'Alonzo et al., 2002; Wagner EF. 2002; MacLean et al., 2003; Hess et al., 2003; Gebhard et al., 2004; Hess et al., 2004; Karreth et al., 2004; Kenner et al., 2004; Papachristou et al., 2006; Goldring et al. 2006). Notably, multiple AP-1 family members show interaction with type X collagen gene and regulate its expression both *in vitro* and *in vivo*. It was previously shown that there is a PTH/PTHrP responsive element along with multiple AP-1 sites in the human *COL10A1* enhancer. c-fos may mediate the suppressive effect of PTH/PTHrP on type X collagen expression via this enhancer (Riemer et al., 2002). Intriguingly, multiple putative AP-1 binding sites were also identified within the murine *Col10a1* enhancer (Chambers et al., 2002; Gebhard et al., 2004). One of which was shown to be functionally active and specific for reporter activity in hypertrophic chondrocytes *in vitro* (Gebhard et al., 2004). However, these reported AP-1 binding sites are located outside and downstream of the 150-bp enhancer that we have demonstrated in transgenic studies (Zheng et al., 2009). We also performed sequence analysis of this 150-bp enhancer and identified two putative AP-1 sites within its 5′- and 3′-ends. We then generated two reporter constructs in which the *LazZ* gene was driven either by the concatenated AP-1 sequences or by the same 150-bp enhancer but with the two putative AP-1 sites deleted. Interestingly, tissue-specific reporter activity was only observed in transgenic mouse embryos in which the two AP-1 sites were deleted, but not the one with the concatenating AP-1 elements (Zheng et al., 2009). Apparently, these data point to a silencing activity of these AP-1 elements, which would be consistent with previous reports on the repressive activity of c-fos on *Col10a1* (Thomas et al., 2000; Gebhard et al., 2004). This result also indicated the existence of other strong transactivators that may bind the enhancing sequences other than the AP-1 sites.

Transcription factor Runx2 and *Col10a1*

Runx2 is a known master transcription factor (TF) that is essential for osteoblast differentiation. Previous studies have shown that *Runx2* null mice completely lack bone formation and die at birth due to respiratory failure (Komori et al., 1997; Otto et al., 1997). Runx2 is also a critical regulator for chondrocyte maturation as demonstrated by both *in vitro* and *in vivo* studies (Kim et al., 1999; Inada et al., 1999; Takeda et al., 2001 and Hinoi et al., 2006). For more than a decade, accumulative data have demonstrated that Runx2 regulates hypertrophic chondrocyte-specific type X collagen gene expression in different species. In Zebrafish, two Runx2 isoforms are involved in transactivation of *Col10a1 in vitro* through its conserved Runx2 binding sites within the distal promoter (Simões et al., 2006). Meanwhile, a Runx2 binding site within the chicken *Col10a1* promoter is responsible for transactivation of Runx2 which is essential for Wnt/beta-catenin signaling to induce chondrocyte hypertrophy (Dong et al., 2006). More recently, a core element (termed the hypertrophy box) responsive to RUNX2, was found to be located in the human *COL10A1* proximal promoter. This cis-element mediates upregulated human *COL10A1* promoter activity in vitro (Higashikawa et al., 2009).

As to murine *Col10a1*, we have previously shown that a 4-kb murine *Col10a1* proximal promoter can only direct weak reporter expression in lower hypertrophic chondrocytes. This 4-kb promoter contains two conserved Runx2 binding sites which contributed to its promoter activity both *in vitro* and *in vivo* (Zheng et al., 2003). We have also demonstrated that the 150-bp *Col10a1* CIS-enhancer is able to mediate high-level tissue-specific reporter expression *in vivo* (Zheng et al., 2009). Interestingly, detailed sequence analysis identified two tandem repeat Runx2 sites within the 3′-end of this 150-bp region: TGTGG***G***-TGTGG***C*** (−4187 to −4176). Candidate EMSA (Electrophoretic mobility shift assay) assay and ChIP (chromatin immunoprecipitation) experiment using Runx2 antibody confirmed the direct interaction of Runx2 with the cis-element containing these tandem-repeat Runx2 sites. More importantly, our transgenic studies demonstrated the *in vivo* requirement of these Runx2 sites in mediating *Col10a1*/reporter expression, since mutating the Runx2 sites in the 150-bp enhancer abolishes its capacity to drive hypertrophic chondrocyte-specific reporter expression (Li et al., 2011). Notably, most of the Runx2 sites, as well as some of the AP1 sites, localized within or outside of the enhancer, are conserved between human and murine type X collagen gene (Fig. 1) (Zheng et al., 2003; Gebhard et al., 2004; Li et al., 2011). There are also Gli1 and Sox9 sites within the

enhancer that are adjacent to the tandem-repeat Runx2 site and have recently been characterized (Leung et al., 2011; Li et al., 2011).

Multiple *Col10a1* regulators

It is well accepted that a spatiotemporal variance in gene expression, the source of tissue-specificity of a cell, is usually controlled via a transcriptional regulatory mechanism (Naef and Huelsken, 2005). Previous studies focused on identification of the single TF that is responsible for such controlling. However, there is growing evidence which suggests that tissue-specific gene expression is regulated by one or two key regulators, or a master TF. This key TF (or TFs) may work with other TFs and co-factors together to confer the tissue-specificity of a gene (Myšičková and Vingron , 2012). As to mouse *Col10a1* gene, Runx2 has been demonstrated as an indispensible regulator (Zheng et al., 2003; Li et al., 2011). Meanwhile, multiple transcriptional regulators (AP1, PTH/PTHrP, SP3/SP1, GADD45β, MEF-2C, HIF-2a, SOX9, HDAC inhibitors, and Carbonic anhydrase (CA) IX etc.) and signaling pathways (Ihh, Wnt or Bmp etc.) have been shown to contribute to *Col10a1* expression (Riemer et al., 2002; Adams et al., 2003; Schipani and Provot, 2003; Dong et al., 2005; Magee et al., 2005; Ijiri et al., 2005; Arnold et al., 2007; Sakimura et al., 2007; Saito et al., 2010; Tsuchimochi et al., 2010; Leung et al., 2011; Dy et al., 2012; Maruyama et al., 2013). Not surprisingly, most of above factors/pathways have been characterized and show certain relations with Runx2 in controlling *Col10a1* expression and chondrocyte hypertrophy (Ijiri et al., 2005; Zhou et al., 2006; Zheng et al., 2009, Hattori et al., 2010; Leung et al., 2011; Li et al., 2011; Dy et al., 2012; Hovhannisyan et al., 2013). GADD45β, an essential mediator for MMP-13 expression, has been shown to work synergistically with Fra2 and Runx2 during terminal chondrocyte differentiation (Ijiri et al., 2005). Sox9, a master transcription factor for cartilage development, is a known negative regulator and has been shown to functionally dominant over Runx2 during endochondral ossification (Zhou et al., 2006, Hattori et al., 2010). Interestingly, a recent study has shown the concomitant transactivation and repression by Sox9 on chonrocytic stage-specific gene expression, including *Col10a1* (Leung et al., 2011). It was also suggested that Sox9 may coordinate with Gli factors to achieve such transcriptional control of target genes, as conserved Gli and Sox9 binding sites were identified within this region (Leung et al., 2011). We notice that these putative Gli and Sox9 sites are adjacent to the tandem-repeat Runx2 sites that are required for tissue-specific *Col10a1* enhancer activity (Li et al., 2011), suggesting a potential interaction between these *Col10a1* regulators.

Intriguingly, we have shown that Runx2 interaction with *Col10a1* cis-enhancer is required but not sufficient for its cell-specific promoter activity, suggesting existence of additional *Col10a1* regulators (Li et al., 2011). The refine-mapped 150-bp *Col10a1* enhancer allows us to search for its transcription factor binding sites (TFBS) using web-based software. TRAP (transcription factor affinity prediction) is a web tool that provides affinity-based ranking of TFs with a p-value (Thomas-Chollier et al., 2011). We used the TRAP program and identified multiple candidate *Col10a1* enhancer-interacting factors, including Hoxa3, Mef2, Gbx1, OG2, NFATc1, Gli1, S8, and Runt family members etc. We notice that, while there are known or putative *Col10a1* transactivators, such as Runx2, Mef2, and S8 (Zheng et al., 2003; Arnold et al., 2007; Imabuchi et al., 2011; Li et al., 2011), NFATc1 has been shown to suppress *Col10a1* expression (Zanotti and Canalis, 2013). Although it is unclear about the mechanism, NFAT proteins did form co-operative complexes with AP-1 family members that are known to be associated with Runx2 in controlling *Col10a1* expression (Penolazzi et al., 2011). The

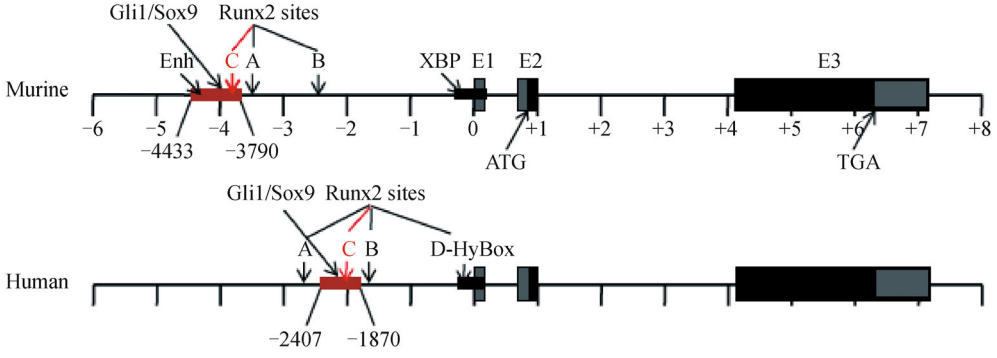

Figure 1 Runx2 sites in murine and human *COL10A1* gene promoter. Illustrated are murine (top) and human (bottom) *COL10A1* gene structure and their promoter/enhancer region. The type X collagen gene contains three exons: E1, E2, and E3. The black bars (XBP) represent type X collagen gene basal promoter, while the red bars (Enh) show the position of murine and human *COL10A1* enhancers (Riemer et al., 2002; Gebhard et al., 2004; Zheng et al., 2009). There are multiple Runx2 sites that locate within the enhancer or promoter region. These Runx2 sites are conserved between murine and human *COL10A1* promoter (A and B sites) or enhancer (C site) (Zheng et al., 2003; Li et al., 2011). Additional Runx2 site is found within the human *COL10A1* basal promoter (D site) (Higashikawa et al., 2009). The functional Gli1 and Sox9 sites within the enhancer are as illustrated (Leung et al., 2011).

above findings suggest that Runx2 is a key *Col10a1* regulator. Multiple other transactivators, as well as repressors, may directly or indirectly work with Runx2 together to regulate cell-specific *Col10a1* expression (Fig. 2).

Additional perspectives of *Col10a1* expression

In general, type X collagen is abundant in hypertrophic chondrocytes and is important for normal distribution of matrix vesicles and proteoglycans within the growth plate. Type X collagen deficiency impairs the supporting properties of the growth plate and the mineralization process, resulting in abnormal long bone phenotypes (Gomez et al., 1996; Kwan et al., 1997; Mäkitie et al., 2010). Type X collagen gene expression may also have some relevance with craniofacial skeletal development and disease. Craniofacial membrane bones are derived from neural crest (NC) cells, which interact with epithelium and undergo cellular condensation to become osteoblasts. Notably, chondrogenic potential and cartilage markers (type II and type X collagen) are reported in calvarial/craniofacial membrane bones (Fang and Hall, 1997). Meanwhile, studies on cranial suture development indicated that Sox9, a master transcription factor for cartilage development, plays an essential role in closure of the posterior frontal suture through endochondral ossification (Sahar et al., 2005). It is not clear yet regarding the contribution of type X collagen during chondrogenesis of membrane bones. However, the above findings, as well as the decreased *COL10A1* expression in cleidocranial dysplasia due to haploinsufficiency of RUNX2, suggest a possible role of type X collagen in intramembranous bone development, as Runx2 is key *Col10a1* regulator, while SOX9 negatively regulates chondrocyte terminal differentiation and *Col10a1* expression during endochondral bone formation (Zhou et al., 2006; Hattori et al., 2010; Leung et al., 2011).

We also notice another finding about increased *COL10A1* expression and cancer formation, especially in the invasive breast cancer cells and tumor samples (Chang et al., 2009; Desmedt et al., 2012; Chapman et al., 2012). However, whether the tumor microenvironment-specific expression of type X collagen constitutes a novel target for the diagnosis and treatment of diverse solid tumor types needs further investigation. More attention may also be needed regarding the possible epigenetic control of type X collagen gene. Previously, multiple CpG methylation sites were identified in human *COL10A1* promoter. Demethylation of two of the CpG sites was found to correlate with induction of *COL10A1* expression during chondrogenesis of mesenchymal stem cells (Zimmermann et al., 2008). Most recently, a microRNA, Hsa-miR-148a, was shown to suppress *COL10A1* expression and hypertrophic differentiation through direct interaction with *COL10A1* and other relevant genes (Vonk et al., 2014).

In summary, we and others have demonstrated the indispensible role of Runx2 in regulation of type X collagen gene expression across species (Drissi et al. 2003; Zheng et al., 2003; Simões et al. 2006; Higashikawa et al. 2009; Zheng et al., 2009; Li et al., 2011). A diversified mechanism has also been suggested as multiple cooperative and repressive elements, in addition to Runx2 sites (and epigenetic control events), within the 150-bp cis-enhancer have been identified (Li et al., 2011). Further characterization of this short cis-enhancer will eventually lead to identification of known or novel *Col10a1* regulators. It is still inconclusive regarding the role of type X collagen with multiple skeletal diseases. As a product of hypertrophic chondrocytes, collagen X expression may be a readout of disease consequence, instead of being the cause of the disease, which is usually more upstream in the chondrocyte differentiation. However, given their specific association with chondrocyte hypertrophy, a critical process during skeletal development and disease, *Col10a1* trans-activators (such as Runx2, Hox3a1 etc.) are expected to promote chondrocyte hypertrophy and bone formation, and are targets for low bone growth as seen in skeletal dysplasia. Meanwhile, although there are no indications that a lack of

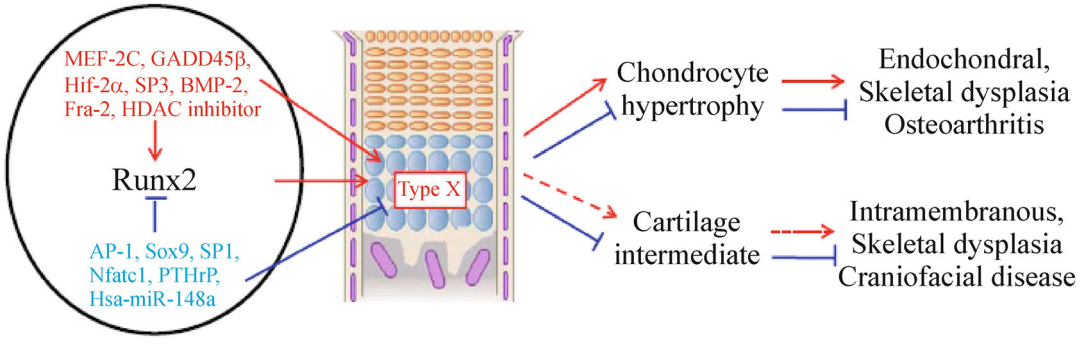

Figure 2 Putative *Col10a1* regulatory mechanism. The key TF, Runx2, work with other TFs together to control hypertrophic chondrocyte-specific *Col10a1* gene expression. Listed are partial TFs which include both transactivators (MEF-2C, GADD45β, Hif-2α, SP3, BMP-2, Fra-2, and HDAC inhibitor etc.) and repressors (AP1, Sox9, NFATc1, pTHrP, Hsa-miR-148a etc.). Molecular regulators controlling the level of type X collagen expression will impact the process of chondrocyte hypertrophy, a process critical for endochondral bone formation, skeletal dysplasia, and osteoarthritis. Red arrow: promote; blue line: inhibit.

type X collagen is protective for OA, the specific *Col10a1* repressors (such as Sox9, NFATc1 etc.) will decrease *Col10a1* expression. This will change the local matrix environment and impair the process of chondrocyte hypertrophy and, therefore, constitute potential therapeutic targets for bone over growth as seen in osteophyte formation in OA.

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Compliance with ethics guidelines

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