The role of histone deacetylase 4 during chondrocyte hypertrophy and endochondral bone development

Chondrocyte hypertrophy represents a crucial turning point during endochondral bone development. This process is tightly regulated by various factors, constituting a regulatory network that maintains normal bone development. Histone deacetylase 4 (HDAC4) is the most well-characterized member of the HDAC class IIa family and participates in different signalling networks during development in various tissues by promoting chromatin condensation and transcriptional repression. Studies have reported that HDAC4-null mice display premature ossification of developing bones due to ectopic and early-onset chondrocyte hypertrophy. Overexpression of HDAC4 in proliferating chondrocytes inhibits hypertrophy and ossification of developing bones, which suggests that HDAC4, as a negative regulator, is involved in the network regulating chondrocyte hypertrophy. Overall, HDAC4 plays a key role during bone development and disease. Thus, understanding the role of HDAC4 during chondrocyte hypertrophy and endochondral bone formation and its features regarding the structure, function, and regulation of this process will not only provide new insight into the mechanisms by which HDAC4 is involved in chondrocyte hypertrophy and endochondral bone development, but will also create a platform for developing a therapeutic strategy for related diseases.

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Article focus
- The role of histone deacetylase 4 (HDAC4) during chondrocyte hypertrophy and endochondral bone development.

Key messages
- HDAC4 represses chondrocyte hypertrophy and endochondral bone formation by inhibiting the function of myocyte-specific enhancer factor 2C (MEF2C) and runt-related transcription factor 2 (RUNX2).
- New breakthroughs might be achieved by controlling microRNA (miRNA), post-translational modifications (PTMs), or cleavage of HDAC4 to regulate chondrocyte hypertrophy and endochondral bone formation.

Strengths and limitations
- This article not only reviews the role of HDAC4 during chondrocyte hypertrophy and endochondral bone development, but also systematically introduces the structural basis and regulatory mechanism of HDAC4.
- Although the authors have tried their best to retrieve the relevant articles in recent years, there may still be some relevant content not included in this article.

Introduction
Endochondral bone formation is critical in vertebrate skeletal development, including in the long bones and vertebrae, via successive steps of mesenchymal condensation, chondrogenesis, chondrocyte maturation, hypertrophy, and finally vasculogenesis and osteoblast recruitment. Chondrocyte hypertrophy is essential for vascular invasion, osteoblast differentiation, and endochondral ossification and represents a crucial turning point from...
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Chondrocyte differentiation to bone formation. Anomalous regulation of chondrocyte hypertrophy disrupts the normal differentiation programme, resulting in a distorted growth plate architecture that leads to skeletal dysplasia with pronounced limb shortening. However, a systematic review regarding how HDaC regulates endochondral bone formation has been lacking. This review focuses on the mechanism by which HDaC is involved in chondrocyte hypertrophy based on its structure, function, and regulation.

Histone deacetylase 4 (HDaC4) is a member of the HDaC class IIa family (HDaC-4, -5, -6, -7, and -10) that promotes chromatin condensation and represses transcription by acetylating lysine to inhibit histone–DNA, histone–histone, and other protein–DNA interactions. These interactions have evolved into different signalling networks of development in different tissues, including adult tissues that adapt to environmental changes. Initial studies on HDaC4 addressed skeletal muscle development, and HDaC4 was found at high levels not only in skeletal muscle but also in the brain, heart, vasculature, cartilage, and liver. The study has shown that mice lacking HDaC4 display a remarkable phenotype characterized by inappropriate chondrocyte hypertrophy that leads to ectopic bone formation. Furthermore, overexpression of HDaC4 in proliferating chondrocytes in vivo inhibits hypertrophy and, thus, ossification of developing bone. These results have established that by controlling chondrocyte hypertrophy, HDaC4 acts as a central regulator of growth plate development.

In addition, recent studies have discovered that during osteoarthritis (OA), chondrocytes lose their stable phenotype and undergo hypertrophic differentiation. Cartilage degeneration in OA is therefore similar to cartilage degradation in the growth plate. Decreases in HDaC4 contribute, at least in part, to the pathogenesis of OA cartilage degeneration. Overall, an understanding of the role of HDaC4 during chondrocyte hypertrophy and endochondral bone formation might provide a new therapeutic strategy for OA.

In this review, we focus on the role of HDaC4 in regulating chondrocyte hypertrophy and endochondral bone formation. The review not only provides new insight into the mechanisms of HDaC4 in chondrocyte hypertrophy and endochondral bone development but also creates a platform for developing a therapeutic strategy for related diseases, including skeletal dysplasias such as pronounced limb shortening and OA.

HDaC4 suppresses chondrocyte hypertrophy and endochondral bone formation by inhibiting the function of MEF2C and RUNX2

During endochondral bone development, chondrocyte hypertrophy is tightly regulated by various factors. The most well-known factors include secreted factors Indian hedgehog (Ihh), parathyroid hormone-related protein (PTHrP), type-X collagen (Col-X), matrix metalloproteinase-13 (MMP-13), vascular endothelial growth factor (VEGF), and transcription factors runt-related transcription factor 2 (RUNX2) and myocyte-specific enhancer factor 2C (MEF2C) (Figure 1). Ihh is a member of the Hedgehog family including sonic hedgehog (Shh), Desert hedgehog (Dhh), and Ihh, which are mainly produced and secreted by prehypertrophic chondrocytes, suggesting a critical role for the Ihh-PTHrP regulatory axis in chondrocyte proliferation.
and differentiation. Studies have found that Ihh stimulates proliferating chondrocytes to produce PTHrP, which in turn accelerates the proliferation of periarticular cells and prevents the onset of chondrocyte hypertrophy, maintaining chondrocytes in a proliferating state. This negative feedback loop regulates the balance between proliferation and maturation of chondrocytes, ensuring orderly bone formation. Furthermore, Kobayashi et al. discovered that Ihh stimulates periarticular chondrocyte differentiation to regulate growth plate length independently of PTHrP.

PTHrP is a member of the parathyroid hormone (PTH) family. It was noted by Albright in 1941, and it has since been found in many tissues. PTHrP plays a key role during endochondral ossification; it is expressed by peri-chondrial cells and early proliferating chondrocytes and then diffuses to act on PTH/PTHrP receptor-bearing cells to inhibit expression of RUNX2 and suppress chondrocyte hypertrophy. In addition to regulating the proliferation and differentiation of chondrocytes by limiting Ihh expression, a recent study showed that PTHrP signalling promotes HDaC4 localization to the nucleus and increases HDaC4-induced inhibition of chondrocyte hypertrophy.

Col-X is secreted by hypertrophic chondrocytes. MMP-13 is a matrix metalloproteinase involved in the degradation of the extracellular matrix molecule type-II collagen. VEGF, an important mediator of endochondral ossification expressed by hypertrophic chondrocytes, is required for chondrocyte survival, cartilage angiogenesis, and endochondral bone development. All these factors are considered to be major biological markers of chondrocyte hypertrophy during skeletogenesis.

RUNX2, also named core binding factor alpha 1 (Cbfα1), polyoma enhancer binding protein 2 alpha 1 (PEBP2aα1), and acute myeloid leukemia gene-3 (AML3), is a member of the runt family of transcription factors that was originally isolated on the basis of its ability to activate transcription of the osteoblast-specific osteocalcin gene. Studies have shown that loss of RUNX2 function slows bone development and that gain of RUNX2 function accelerates bone formation, with an important role in maintaining the balance between proliferation and hypertrophy in chondrocytes. During endochondral bone formation, RUNX2 not only regulates expression of Col-X and MMP-13 in chondrocytes but also activates the Ihh promoter and stimulates Ihh expression, participating in the Ihh/PTHrP negative feedback loop. Most importantly, HDaC4 directly inhibits the function of RUNX2 to control chondrocyte hypertrophy during skeletogenesis.

MEF2 is a MADS (MCM1, Agamous, Deficiens, Serum response factor) box factor implicated in muscle and cardiovascular development. There are four mammalian MEF2 genes: MEF2A, MEF2B, MEF2C, and MEF2D. MEF2C was shown to be necessary for chondrocyte hypertrophy, and knocking it down inhibits expression of RUNX2, Col-X, and VEGF. Thus, MEF2C acts upstream of RUNX2 during chondrocyte hypertrophy, and MEF2C promotes RUNX2 expression and chondrocyte hypertrophy. HDaC4 repression in chondrocyte hypertrophy is mediated by MEF2C transcription factors.

All of the factors mentioned above constitute a complex and regulatory network that maintains normal bone development. HDaC4 is involved in the network by interacting directly with MEF2C and RUNX2 and inhibiting their expression and function, thereby suppressing chondrocyte hypertrophy and endochondral bone development. HDaC4 is believed to be a central regulator in the network.

Structure of the HDaC4 protein

HDaC4 has two distinct domains. The highly conserved carboxyl-terminal (C-terminal) domain of approximately 400 to 450 amino acids (residues 648 to 1057) with a hydrophobic nuclear export sequence (NES, 1051 to 1084) enables HDaC4 to shuttle between the nucleus and cytoplasm. The long amino-terminal (N-terminal) domain of approximately 450 to 600 amino acids includes a nuclear localization signal (NLS, 247 to 285) that is related to its ability to shuttle between the cytoplasm and nucleus. The C-terminus mainly mediates HDaC4 function. The N-terminus is believed to be the key feature that distinguishes class I HDAC proteins; it is involved in interacting with different transcription factors to exert specific functions in different contexts. Many HDaC4 partners have been identified, such as MEF2C, vega et al. found that the ruNX2 interaction with HDaC4 is localized within the first 220 amino acids of the protein, which also coincides with the MeF2-binding region. Further studies in vitro demonstrated that HDaC4 binds to Cbfα in a Ca2+-dependent manner via amino acids 150 to 220 of HDaC4 and that Cbfα competes for HDaC4 with MEF2C, and studies have shown that MEF2C binds to amino acids 118 to 180 of HDaC4. Studies have shown that HDaC4 2aa to 201aa binds to srF with greater affinity than does HDaC4 2aa to 201aa, suggesting that the region between 201aa and 289aa contains a critical part of the SRF-binding domain (Figure 2).
Function of the HDAC4 protein

Studies indicate that HDAC4 exhibits very little or no deacetylase activity, similar to the other members of the HDAC class IIa protein family. Indeed, only upon recruiting HDAC3, nuclear-hormone receptor corepressor (NcoR), and silencing-mediator-repressor-transcription (SMRT) to form the HDAC4-HDAC3/NcoR/SMRT complex does HDAC4 engage in enzymatic activity.43 In other words, HDAC4 is mainly responsible for recruitment in the deacetylase process. During chondrocyte hypertrophy and endochondral bone development, the HDAC4 N-terminus can also recruit target proteins through protein–protein interactions.

Inhibiting chondrocyte hypertrophy and endochondral bone development by binding and suppressing transactivation of MEF2C. MEF2 was initially identified as a transcription factor that activates expression of skeletal and cardiac muscle structural genes.32 Later, MEF2 was reported to be expressed in lymphocytes, the neural crest, smooth muscle, endothelium, and bone, and it plays central roles in diverse developmental programmes.44 MEF2C is one of the isoforms of MEF2; as mentioned earlier, it is a key regulator during chondrocyte hypertrophy and endochondral bone development through regulation of the function of Col-X, RUNX2, Ihh, and VEGF.33,45 Studies have demonstrated the affinity of HDAC4 for MEF2C in vitro and in vivo, showing that their interaction can inhibit chondrocyte hypertrophy by suppressing MEF2C transactivation.46 Binding of CaM to HDAC4 leads to HDAC4 dissociation from MEF2, relieving MEF2 from the transcriptional repression induced by HDAC4.38 Therefore, the authors of the study contended that the balance between the opposing actions of MEF2C and HDAC4 have a key function in chondrocyte hypertrophy and bone formation.38 However, research on the mechanism by which HDAC4 suppresses MEF2C in chondrocytes and bone formation is scarce. We review three different reported strategies by which HDAC class IIa proteins influence MEF2C transcription: 1) DNA-bound HDAC4 recruits HDAC class IIa proteins, and local chromatin is deacetylated by HDAC class IIa proteins through interaction with the N-terminus of HDAC class IIa proteins, ultimately repressing MEF2C transcriptional activity;47 2) HDAC4 suppresses MEF2C transcription activity by promoting the sumoylation of MEF2C through association of the N-terminal domain with corepressors such as CtBP;48 3) the N-terminal HDAC4 cleavage product, HDAC4-NT, accumulates in the nucleus where it interacts with MEF2 to inhibit its activity in cardiomyocytes.49 These findings are expected to be useful in future research on the mechanism of HDAC4-mediated suppression of MEF2C in chondrocytes and bone formation.

Inhibiting chondrocyte hypertrophy and endochondral bone development by binding and suppressing transactivation of RUNX2. RUNX2 is necessary for mesenchymal condensation, osteoblast development, chondrocyte hypertrophy, vascular invasion of the developing skeleton, and activation of bone marrow endothelial cell migration and invasion.50 RUNX2 exerts its function both alone and in conjunction with numerous proteins to integrate a variety of signals and organize gene expression.51 HDAC4 directly inhibits the function of RUNX2 to control chondrocyte hypertrophy during skeletogenesis.11 However, the interactions of HDAC4 and RUNX2 during chondrocyte hypertrophy remain mechanistically and functionally poorly characterized. The first potential explanation is that HDAC4 specifically perturbs associations of RUNX2 with its target sequence in chromatin and suppresses acetylation of histone H3 on the RUNX2 promoter.51 In the second reported mechanism, HDAC4 directly deacetylates RUNX2 and RUNX3 to initiate their ubiquitin-mediated degradation.52 Interestingly, it was recently...
discovered that the RUNX2-induced osteoblast differentiation programme of C3H10T1/2 cells is unaffected by the nuclear/cytoplasmic location of HDAC4.53

The 14-3-3 protein interacts with HDAC4 to inhibit HDAC4 translocation into the nucleus to prevent HDAC4 inhibition of targeted proteins. Nucleocytoplasmic transport is considered to be an important process for controlling the transcription corepressor function of HDAC4 because, as researchers have argued, HDAC4 localization to the nucleus is necessary to repress transcription.34 The 14-3-3 protein interacts with HDAC4 via S246, S467, and S632 phosphoserines to escort phospho-HDAC4 from the nucleus to the cytoplasm, thus relieving HDAC4-induced inhibition of target proteins. It is well known that calcium/calmodulin-dependent protein kinase (CaMK)-I, II, and -IV preferentially phosphorylate S246 or S467 and S632 of HDAC4 to promote its nuclear export and interaction with the 14-3-3 protein; the decreased level of nuclear HDAC4 inhibits its ability to repress the transcription of targets.35 Recently, a study showed that PTHrP signalling induces HDAC4 to separate from the 14-3-3 protein and migrate to the nucleus to inhibit chondrocyte hypertrophy through decreased phosphorylation of the 14-3-3-binding sites of HDAC4.45 Furthermore, SIK3 is reported to be essential for chondrocyte hypertrophy during skeletal development in mice, and the mechanism is similar to that of the 14-3-3 protein: SIK3 binds to HDAC4 and prevents it from leaving the cytoplasm.40

All of the above results indicate that HDAC4 recruits its target proteins via its N-terminus and that it forms the HDAC4-HDAC3/NCOR/SMRT complex to exert its deacetylase function on target proteins via its C-terminus. The most interesting finding is that HDAC3 is located mainly in the nucleus, which means HDAC4 is located in the nuclei and might be necessary for HDAC4 to work. This can explain why interaction between HDAC4 and the 14-3-3 protein prevents HDAC4 inhibition of targeted proteins. The hypothesis needs more work to be verified.

Regulation of the HDAC4 protein

The function of HDAC4 depends on its subcellular location, which is regulated by miRNA, post-translational modification (PTM), cleavage, the 14-3-3 protein, and mechanical loading during chondrocyte hypertrophy and endochondral bone formation.

miR-1 and miR-365 inhibit expression of HDAC4 during chondrocyte hypertrophy and endochondral bone formation. MicroRNAs (miRNAs) play a crucial role in the genetic regulation of HDAC4. To date, approximately 20 miRNAs have been discovered that regulate HDAC4 expression in different tissues, namely: miR-1,54,55 miR-133,54 miR-365,56 miR-206,57,58 miR-206-3p,59 miR-22,60 miR-140,61 miR-125a-Sp,62 miR-155,63 miR-20a-Sp,64 miR-548ah,65 miR-520b,66 miR-145-3p,67 miR-378a-3p,68 miR-381,59 miR-9,70 miR-483-Sp,71 miR-29a,72 miR-200a,73 and others (Table I). Of these miRNAs, miR-1 and miR-381 are reported to be specifically expressed in growth plate cartilage and to regulate the chondrocyte phenotype by suppressing HDAC4 expression during growth plate development.74,69 Expression of miR-365 is elevated in the prehypertrophic zone of the growth plate, which stimulates chondrocyte differentiation by targeting HDAC4.75

Post-translational modifications regulate the subcellular localization of HDAC4. Regarding regulation at the protein level, PTMs are believed to mainly control spatial and temporal histone deacetylase functions by regulating subcellular localization. These PTMs include

**Table I. MicroRNAs targeting histone deacetylase 4.**

| miRNA  | Function                                                                 | References |
|--------|--------------------------------------------------------------------------|------------|
| miRNA-1 | Promoting the differentiation of duck myoblasts; blunting cardiomyocyte hypertrophy elicited by thyroid hormone; regulating chondrocyte phenotype. | 54,55,74  |
| miRNA-133 | Affecting SRF and TGFBR1 expression to promote proliferation of duck myoblasts. | 54          |
| miRNA-365 | Ameliorating dexamethasone-induced suppression of osteogenesis, stimulating chondrocyte differentiation. | 56,75,57,58 |
| miRNA-206 | Attenuating denervation-induced skeletal muscle atrophy represses hypertrophy of myogenic cells. | 59,60,61   |
| miRNA-206-3p | Alleviating chronic constriction injury-induced neuropathic pain. | 62,63       |
| miRNA-22 | Promoting Th17 cell differentiation in inflammatory intestinal disease progression. | 64          |
| miRNA-140 | Inhibiting proliferation of osteosarcoma cells. | 65          |
| miRNA-125a-Sp | Suppressing breast tumourigenesis. | 66          |
| miRNA-155 | Impairing transcriptional activity of B-cell lymphoma 6 (BCL6). | 67          |
| miRNA-20a-Sp | Attenuating allergic inflammation in HMC-1 cells. | 68          |
| miRNA-548ah | Promoting the replication and expression of hepatitis B virus. | 69          |
| miRNA-520b | Restraining cell growth in lung cancer. | 70          |
| miRNA-145-3p | Suppressing proliferation and promotes apoptosis and autophagy of osteosarcoma cell. | 71          |
| miRNA-378a-3p | Promoting differentiation and inhibiting proliferation of myoblasts in skeletal muscle development. | 72          |
| miRNA-381 | Regulating chondrocyte hypertrophy. | 73          |
| miRNA-9 | miR-9 upregulation integrates post-ischemic neuronal survival and regeneration in vitro. | 74          |
| miRNA-483-5p | Regulating HDAC4 mRNA during embryogenesis. | 75          |
| miRNA-29a | Modulates the profibrogenic phenotype of the activated hepatic stellate cells. | 76          |
| miRNA-200a | Downregulation of miR-200a enhances the proliferation and migration of HCC cells. | 77          |

HCC, hepatocellular carcinoma; HDAC4, histone deacetylase 4; HMC, human mast cell; miR, microRNA; mRNA, messenger RNA; SRF, serum response factor; TGFBR1, transforming growth factor beta receptor 1; Th17, T-helper 17 cell.
phosphorylations/dephosphorylations, sumoylation, disulfide bond formation, and ubiquitination and proteolytic cleavage.\textsuperscript{15}

Phosphorylation and dephosphorylation of HDAC4 regulate its subcellular localization. Most kinases including CaMK, protein kinase D (PKD), adenosine 5’-monophosphate (AMP)-activated protein kinase (AMPK), salt-induced kinase (SIK), and dual specificity tyrosine-phosphorylation-regulated kinase 1B (DyrKB1) phosphorylate HDAC proteins and enhance their nuclear efflux.\textsuperscript{76,77} The phosphorylation at HDAC4 S246, S467, and S632 can promote HDAC4 movement from the nucleus such that it can interact with the 14-3-3 protein and remain in the cytoplasm; dephosphorylation of these three sites leads to the opposite results. Nonetheless, protein kinase A (PKA) phosphorylates S265 and S266 of HDAC4 in cardiac and skeletal muscle, which results in HDAC accumulation in the nucleus.\textsuperscript{78} A recent study showed that PKA can mediate novel S584 phosphorylation of HDAC4 and thus enhance its function through a mechanism similar to that mediated by phosphorylation of S265 and S266.\textsuperscript{79} Furthermore, members of the protein phosphatase 2A (PP2A) family can remove HDAC4 phosphorylation and promote HDAC4 nuclear translocation to promote its transcriptional-repressive function.\textsuperscript{80}

Sumoylation of HDAC4 regulates the subcellular distribution of HDAC4. HDAC4 is modified at K559 by the ubiquitin-related SUMO-1 modifier, and a sumoylation-deficient point mutant (HDAC4-K559R) slightly impairs its ability to repress transcription and reduces its histone deacetylase activity. During the process, the ability of HDAC4 to self-aggregate is a prerequisite for proper sumoylation in vivo. These findings suggest that sumoylation may be an important regulatory mechanism for controlling the transcriptional repression mediated by HDAC4.\textsuperscript{81} Furthermore, proteasome-dependent degrada
tion of HDAC4 is associated with K559 sumoylation.\textsuperscript{82}

Formation of disulfide bonds promotes HDAC4 translocation from the nucleus. A study showed that reactive oxygen species (ROS)-generating hypertrophic stimuli promote the oxidation of both Cys-274/Cys-276 in DnaJb5 and of C667/C669 in HDAC4 and the formation of intramolecular disulfide bonds, which signal HDAC4 nuclear export and thus inhibit its function.\textsuperscript{83}

Ubiquitination of HDAC4 mediates the degradation of HDAC4. Some studies have shown that the stability of HDAC4 is influenced by its polyubiquitination and degra
dation, and serum and growth factor deprivation induces polyubiquitination and proteasome-mediated degradation of HDAC4 and thus achieves HDAC4 regulation.\textsuperscript{84,85} However, the specific mechanism remains unclear.

**Caspase cleavage and PKA-dependent cleavage of HDAC4.**

There is evidence that caspase 2 and caspase 3 can induce the cleavage of HDAC4 at Asp289.\textsuperscript{86} The N-terminal fragment generated can lead to markedly increased apoptosis via the suppression of RUNX2 action in chondrocytes.\textsuperscript{87,88} Additionally, HDAC4 can be cleaved by PKA too. One study showed that PKA induces HDAC4 cleavage at T201,\textsuperscript{89} a site not found in other HDAC class IIa proteins, by a serine protease that has not yet been identified. This cleavage results in the production of an N-terminal fragment (HDAC4-NT) that accumulates in the nucleus to suppress MEF2 activity, enabling cardiomyocytes to exhibit differential hypertrophy\textsuperscript{90} and/or to protect against heart failure.\textsuperscript{89} Few studies to date have examined the role of the C-terminal fragment of HDAC4.

**Mechanical loading promotes translocation of HDAC4 to the nucleus.** Recent studies have shown that mechanical loading can regulate the function of HDAC4 in chondrocytes, and the results also showed that compressive loading induces HDAC4 relocation from the cytoplasm to the nucleus in chondrocytes via PP2A-dependent HDAC4 dephosphorylation at the sites of S246/S467/S632, which leads to suppression of RUNX2 expression and inhibition of chondrocyte hypertrophy.\textsuperscript{90,91} Moreover, in OA pathogenesis mechanical pressure is reported to downregulate HDAC4 expression by upregulating miR-365, which in turn causes cartilage degradation.\textsuperscript{92}

Regulating the subcellular localization of HDAC4 is one of the important ways to regulate its function. Research into controlling miRNA, PTMs, cleavage of HDAC4, or mechanical loading to regulate the subcellular localization of HDAC4 and chondrocyte hypertrophy and endochondral bone formation may lead to new breakthroughs. Other approaches include mutating the caspase cleavage site to increase HDAC4.

In conclusion, HDAC4 plays a crucial role in chondrocyte hypertrophy and endochondral bone development. This review discusses the role of HDAC4 in the signalling networks regulating chondrocyte hypertrophy and endochondral bone development and the features of HDAC4 with regard to its structure, function, and regulation. In summary, HDAC4 located in the nucleus might be necessary for its function, and new breakthroughs might be achieved by controlling miRNA, PTMs, cleavage of HDAC4, or mechanical loading to regulate chondrocyte hypertrophy and endochondral bone formation.

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This study did not require ethical approval.

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