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Hypoxia-inducible factor 2 α is a novel inhibitor of chondrocyte maturation

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Abstract

Hypoxic environment is essential for chondrocyte maturation and longitudinal bone growth. Although hypoxia-inducible factor 1 alpha (Hif-1 α) has been known as a key player for chondrocyte survival and function, the function of Hif-2 α in cartilage is mechanistically and clinically relevant but remains unknown. Here we demonstrated that Hif-2 α was a novel inhibitor of chondrocyte maturation through downregulation of Runx2 stability. Mechanistically, Hif-2 α binding to Runx2 inhibited chondrocyte maturation by Runx2 degradation through disrupting Runx2/Cbfb complex formation. The Hif-2 α -mediated-Runx2 degradation could be rescued by Cbfb transfection due to the increase of Runx2/Cbfb complex formation. Consistently, mesenchymal cells derived from Hif-2 α heterozygous mice were more rapidly differentiated into hypertrophic chondrocytes than those of wild type mice in a micromass culture system.

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AUTHOR CONTRIBUTIONS

X Che and JY Choi were involved in the design of the experiment, acquisition and analysis of data, writing the draft, and revising the manuscript. JY Choi, NR Park, X Jin, CY Park, SG Kim, HJ Kim, JB Lian, JL Stein, and GS Stein contributed to data interpretation and revision of the manuscript. MS Han and YK Jung were involved in acquisition of data and revision of the manuscript. JS Chun provided the Hif-2 α genetic mice, was involved in data interpretation, and revised the manuscript. J Jin was involved in data interpretation and revision of the revised manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

Collectively, these findings demonstrate that Hif-2 α is a novel inhibitor for chondrocyte maturation by disrupting Runx2/Cbfb β complex formation and consequential regulatory activity.

Keywords

Hif-2 α ; Runx2; Cbfb β ; Proteasomal degradation; chondrocyte maturation

INTRODUCTION

In vertebrates, the skeleton is formed through intramembranous and endochondral ossification. During endochondral ossification, mesenchymal cells condense and differentiate into hypertrophic chondrocytes by induction of key transcription factors such as Sox9 and Runx2 (Komori, 2018; Long & Ornitz, 2013; Stein et al., 2004). To date, Runx2 together with Runx3 have been known as a transcriptional activator of chondrocyte hypertrophy and maturation (Yoshida et al., 2004). Ablation or C-termini truncation of Runx2 leads to the extension of the hypertrophic chondrocyte zone without bone marrow formation (Choi et al., 2001; Komori et al., 1997; Kundu et al., 2002; Otto et al., 1997). In contrast, overexpression of Runx2 in cartilage accelerates chondrocyte maturation and endochondral bone formation lead to short limb formation and enhanced mineralization (Takeda et al., 2001; Ueta et al., 2001). In addition, core binding factor β (Cbfb β), a partner protein of Runx family transcription factors is essential for Runx2 protein stability and transcription activity. Chondrocyte-specific deletion of Cbfb β in mice shows delayed endochondral bone formation with a short appendage skeletal length and increased proliferative chondrocytes (Park et al., 2016). Taken together, Runx2/Cbfb β complex is essential for chondrocyte maturation and longitudinal bone growth.

The hypoxic environment is a prominent feature of the developmental growth plate in mammals, and this hypoxia occurs in the inner layer, not in the outer periphery of cartilage. Hypoxia-inducible factor (Hif)-1 α and Hif-2 α are key transcription factors that respond to hypoxia changes and signaling in chondrocytes (Semenza, 2012). They are all degraded by the proteasome-mediated degradation in normoxic conditions and, conversely, stabilized under hypoxia (Semenza, 2012). Mice deficient in Hif-1 α or Hif-2 α die from vascular defects and impaired erythropoiesis on days 10.5 and 12.5 of embryos, respectively (Iyer et al., 1998; Peng et al., 2000). A previous study has reported that Hif-1 α is essential for chondrocyte growth inhibition and survival (Schipani et al., 2001). In addition, Hif-1 α , conditionally inactivated in the limb bud mesenchyme using a *Prx1* promoter-driven Cre, exhibits early chondrogenesis and joint dysplasia (Araldi et al., 2011). Indeed, Hif-1 α is a positive regulator of Sox9, which is required for chondrocyte differentiation and cartilage formation (Tanimoto et al., 2003). Hif-1 α is a positive regulator of chondrocyte maturation, survival and *in vivo* activity, at least in part of the regulation of collagen synthesis in chondrocytes (Stegen et al., 2019).

Previously, Hif-2 α has been known as a Runx2 upstream regulator in osteoblasts (Tamiya et al., 2008) and is also expressed in the hypertrophic zone of the growth plate and in articular chondrocytes (Stewart et al., 2006). Hif-2 α null mice die in the early embryonic

stage (between E9.5 and E13.5) before cartilage anlage formation with varying phenotypes depending on the genetic background of the mice (Patel & Simon, 2008). Heterozygous Hif-2 α or conditional deletion of Hif-2 α in limb bud mesenchyme causes a temporary and modest delay in endochondral bone development (Araldi et al., 2011; Saito et al., 2010; Yang et al., 2010). However, the Hif-2 α function of chondrocyte differentiation and maturation still controversial. As a catabolic factor, Hif-2 α enhances osteoarthritis or accelerates articular chondrocyte differentiation into hypertrophic chondrocytes (Saito et al., 2010; Yang et al., 2010). Furthermore, Hif-2 α upregulates Sox9 and Trap6, respectively, impairing osteoblast and partially stimulating osteoclast differentiation (Lee et al., 2019; Merceron et al., 2019). Considering that both Runx2 and Hif-2 α are highly expressed in hypertrophic chondrocytes of the growth plate, we hypothesized that Hif-2 α regulates Runx2 during chondrocyte differentiation and maturation. In this study, we found that Hif-2 α blocks Runx2/Cbfb β complex formation, thereby playing an inhibitory role in chondrocyte maturation through accelerated Runx2 proteasomal degradation.

MATERIALS AND METHODS

Animals

Animal care and experiments were performed in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of Kyungpook National University (KNU-2018–0020). Animals were housed 5 per cage in a specific pathogen-free environment on a 12-h light cycle at 22 \pm 2°C with standard chow (Greenpia Technology, Yeosu-si, South Korea) and had free access to tap water.

Antibodies and reagents

Rabbit anti-HA, Rabbit anti-Hif-2 α , goat anti-Runx2, monoclonal anti-actin and goat anti-Lamin B antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Rabbit anti-Myc and monoclonal anti-Runx2 were purchased from Abcam (MA, USA). Monoclonal anti-Flag, monoclonal anti-Flag-HRP, HA-anti HA-agarose antibody and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cells and micromass culture

ROS 17/2.8 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL; NY, USA) containing 10% fetal calf serum (FCS, Gibco BRL) and 1% antibiotics (Lonza, Rockland, ME, USA). ATDC5 cells were cultured in DMEM/F12 (1:1) hybrid medium (Lonza) supplemented with 5% fetal bovine serum (FBS, Gibco BRL), 10 μ g/ml human transferrin (Sigma-Aldrich), and 3 \times 10⁻⁸ M sodium selenite (Sigma-Aldrich). Vascular smooth muscle MOVAS cells (ATCC, VA, USA) were maintained in DMEM containing 10% FBS, 0.2 mg/ml G-418 (sigma) and 1% antibiotics.

For micromass culture (Stanton et al., 2004), primary mesenchymal cells were isolated from limbs of wild type and Hif-2 α ^{+/-} mouse embryos at day 11.5 (E11.5) and digested in 10% (v/v) fetal bovine serum (FBS)/Puck's solution A (0.4 g of KCl/l, 8 g of NaCl/l, 0.35 g of NaHCO₃/l, 1 g of glucose/l) containing 10 mg of dispase/ml (1 unit/ml) for 2 hours at 37 °C. Dispase activity was neutralized with 2:3 DMEM/F12 media containing 10% FBS

(GibcoBRL). The digested cells were filtered through 40 μm cell strainers and centrifuged at 1500 rpm for 3 min to obtain a cell suspension. The cells were resuspended in growth media at 2×10^7 cells/ml and spotted in 10 μl drops/well of a 12-well culture plate. After a 2h attachment, the micro-masses were fed with growth media that consisted of 2:3 DMEM/F12 medium (Sigma-Aldrich) containing 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and 10% FBS. For chondrocyte differentiation, 10 mM β -glycerophosphate and 50 $\mu\text{g/ml}$ ascorbic acid were added to the growth medium for 28 days. Media was replenished with fresh media every two days (Park et al., 2016).

Transient transfection and knock-down experiments

ATDC5, ROS 17/2.8 cells, primary chondrocytes or MOVAS cells were plated at a density of 2×10^5 cells/well on 6 well plates for transfection experiments. Cells were transfected with a total of 4 μg DNA, including 2 μg pCMV-HA-Hif-2 α (Tamiya et al., 2008), 1 μg pcDNA3.1-myc-Cbfb and 1 μg pCS4-2flag-Runx2 by using Lipofectamine 2000 (Invitrogen, CA, USA), and cells were grown for 24 h. All transfections were performed in triplicate. The Sh-Control-GFP and Sh-Hif-2 α -GFP constructs (kindly provided by Dr. Je-Yoel Cho, Seoul National University, Seoul, South Korea) were infected to the ATDC5 cells.

Western blot analysis

Whole cells were lysed by radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 0.5% SDS, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 $\mu\text{g/ml}$ pepstatin, and 1 $\mu\text{g/ml}$ leupeptin), and the protein concentration was measured by Bradford protein assay kit (Bio-Rad, CA, USA). Proteins were separated on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. Membrane was blocked with 5% skim-milk and immunoblotted with primary antibody overnight at 4°C. Proteins were detected by using an enhanced chemiluminescence detection system (GE Healthcare, Uppsala, Sweden).

Total RNA isolation and gene expression analysis

Total RNA was isolated from cells by using TRIzol reagent (Invitrogen) according to the manufacturers' instructions. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by using Superscript II RT (Invitrogen) according to the manufacturer's instructions.

Quantitative real-time PCR was performed using an ABI 7500 real-time PCR system and the relative quantification of *Gapdh* mRNA levels were analyzed by using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA). Primers were designed by using PrimerExpress software (Applied Biosystems). The sequences of primers are as follows: Hif-2, 5'-GCTTCCTTCGGACACATAAGCT-3' and 5'-GAAACCCTCCAAGGCTTTCAG-3'; Runx2, 5'-ACATGGCCAGATTCACAGTGG-3' and 5'-TGGTGCCCGTTAGCAATTG-3'.

Immunofluorescent staining

ATDC5 cells on chamber slide were fixed with 4% paraformaldehyde for 1 min. Cells were permeabilized with 0.1% Triton X-100 and blocked with 1% BSA then incubated with primary antibody for 90 min. After washing the cells 3 times with PBS-T, FITC-conjugated secondary antibodies and DAPI were added to the cells. Fluorescence images were analyzed with a fluorescence microscope (Leica, Wetzlar, Germany). The fluorescence intensity was analyzed by the Leica or image J program.

Immunohistochemistry

E15.5 mice forelimbs were fixed in 4% formaldehyde solution (Junsei, Tokyo, Japan) at 4°C for 24 h. After fixation, the sample was gradually dehydrated in ethanol and embedded in paraffin, and then the paraffin block was sectioned at 3 μm. Deparaffinized sections were quenched in hydrogen peroxide treatment (3% H₂O₂ in 100% methanol, 30 min), retrieved in Tris-EGTA (TEG) buffer (pH 9.0) for 30 min at 60°C oven and incubated in 50 mM ammonium chloride (NH₄Cl) for 30 min at RT. After blocking with 1% BSA, the sections were incubated overnight at 4°C with anti-Runx2 or anti-Cbfb antibodies then incubated for 1 h with the appropriate HRP conjugated anti-IgG antibody then developed using Dako Liquid DAB⁺ substrate-chromogen system (Dako, CA, USA). For histological analysis of micromass culture, cell aggregates were sectioned at day 28, rinsed 3 times in PBS, and fixed in 4% (v/v) paraformaldehyde for 1 h. Specimens were gradually dehydrated in ethanol, embedded in paraffin and sectioned at 3 μm for histological procedures. Images were analyzed with a Leica fluorescence microscope (Leica).

Ubiquitination assay

ATDC5 cells were plated in 100 mm plates at a density of 2×10^6 cells/well then transfected with a total of 12 μg DNA, including 4 μg of the pCMV-HA-Hif-2α (Tamiya et al., 2008), 4 μg of pcDNA3.1-flag-Ubiquitin and 4 μg of pCS4-myc-Runx2 using Lipofectamine 2000, and were grown for 24 h then treated with 20 μM MG132 for 2 h. Whole cells were lysed with lysis buffer [20 mM HEPES (Sigma-Aldrich), pH 7.9, 300 mM KCl (Sigma-Aldrich), the 10% NP-40 (Sigma-Aldrich), 1mM DTT(Invitrogen) and proteinase inhibitors]. Cell lysates (400 μg) were bound with anti-Myc antibody overnight at 4 °C with gentle agitation, then 0.1 g/ml of Separose A beads (GE Healthcare) were incubated in cell lysates at 4 °C for 2 h with gentle stirring. The mixture was washed with lysis buffer. Proteins were eluted by boiling in 50μl protein loading buffer. Ubiquitination levels were confirmed by Western blotting with anti-Flag antibody.

Co-IP assay

ATDC5 cells were plated at a density of 2×10^6 cells/well in 100 mm plates for transfection experiments. Cells were transfected with a total of 6 μg DNA, including 2 μg of the pCMV-HA-Hif-2α (Tamiya et al., 2008), 2 μg of pcDNA3.1-myc-Cbfb or 2 μg of pCS4-2Flag-Runx2 or pCS4-2Flag-delta Runx2 (Runx2 Runt domain deleted gene (Lu et al., 1995) [Kindly provided by YOSHIKI ITO, Cancer Science Institute of Singapore, Singapore] cloned to pCS4-2Flag) by using Lipofectamine 2000 (Invitrogen), and were grown for 16 h. Total cell were lysed with lysis buffer (150mM Sodium chloride, 10mM Tris pH 7.4, 1

mM EDTA, 1mM EGTA, pH 8.0, 1 % Triton-X, 0.5 % NP 40, 2 Mm sodium ortho-vanadate and proteinase inhibitor). Protein concentration was measured by Bradford protein assay kit. Cell lysates (400 µg) were bound with rabbit anti-Myc (Abcam), monoclonal anti-Flag (Sigma-Aldrich) or rabbit anti-HA (Santa Cruz) antibody overnight at 4 °C with gentle agitation then 0.1 g of Separose A beads were incubated in lysis buffer at 4 °C for 2 h with gentle stirring. The mixture was washed with lysis buffer for 5 times. Proteins were eluted by boiling in 50 µl protein loading buffer. Co-IP was confirmed by western blot with anti-Flag or anti-Myc antibody.

Alcian Blue staining

Chondrocyte differentiation was detected by Alcian Blue staining. After washing 3 times with the PBS, micromass sections were fixed in 4% formalin for 1 h at RT. After adding 1% Alcian blue for 1 h, the sections were rinsed with 0.1M HCl then washed with distilled water.

Von Kossa staining

Calcium deposition was detected by von Kossa staining. After washing 3 times with the PBS, micro-mass sections were fixed in 4% formalin for 1 h at RT. After adding 1% silver nitrate around 5 min under long wave ultraviolet light, the sections were thoroughly rinsed with distilled water.

Statistical analyses

Statistical analyses were performed using Sigma Plot software 10.0 (Systat Software Inc, Chicago, USA). Data are expressed as mean ± SD. Difference between groups means were assessed by Student's t-test. * $p < 0.05$ was considered statistically significant.

RESULTS

Hif-2 α inhibits Runx2 protein expression in chondrocytes

Runx2 has been known as a positive regulator for chondrocyte maturation. To investigate Hif-2 α if could affect the Runx2 expression, we first examined Runx2 protein level after transient transfection with Hif-2 α in osteoblasts and chondrocytes. As previously reported (Tamiya et al., 2008), overexpression of Hif-2 α increased Runx2 expression in osteoblast-like ROS 17/2.8 cells (Figure 1A). However, in the chondrogenic cell line ATDC5, Runx2 protein levels were dose-dependently reduced by Hif-2 α (Figure 1B). In addition, overexpressed Hif-2 α reduced Runx2 and Mmp13 expression at various time points in ATDC5 (Figure 1C) as well as primary chondrocytes (Supplementary Figure 1A–B). More interestingly, vascular smooth muscle MOVAS cell line endogenously expressed Hif-2 α but not Runx2, and forced expression of Runx2 or Hif-2 α did not affect each other (Supplementary Figure 1C). Conversely, knockdown of Hif-2 α with Hif-2 α -specific shRNA increased Runx2 (Figure 1D). However, overexpression or knockdown of Hif-2 α did not affect Runx2 mRNA expression in the chondrocytes (Figure 1E and F). Moreover, Hif-2 α reduced Runx2 expression in the nucleus and knockdown of Hif-2 α by Sh-Hif-2 α increased Runx2 in chondrocytes (Figure 2A–D). These results indicate that Hif-2 α inhibits Runx2 protein expression at the post-transcriptional level in chondrocytes.

Hif-2 α enhances Runx2 polyubiquitination

To elucidate the molecular mechanism of Hif-2 α -mediated post-transcriptional Runx2 inhibition, Runx2 polyubiquitination assays were performed in ATDC5 cells by transfection with HA-Hif-2 α , Myc-Runx2 and Flag-Ub expression vectors with or without MG132. Hif-2 α significantly enhanced Runx2 polyubiquitination compared to MG132-treated control cells, indicating that Hif-2 α accelerated Runx2 degradation via Runx2 polyubiquitination (Figure 3A). Our previous studies have shown that Runx2 post-transcriptional stability increases chondrocyte differentiation and maturation and osteoblast differentiation primarily due to the formation of a complex with Cbfb in chondrocytes and osteoblasts. Cbfb has no nuclear localization signal, and translocation of Cbfb into the nucleus mediated by heterodimerization with Runx2 upregulates Runx2 transcription activity in the chondrocytes and osteoblasts (Lim et al., 2015; Park et al., 2016). To test whether Hif-2 α can affect the cellular distribution or expression level of Cbfb, Hif-2 α or Hif-2 α shRNA were expressed in ATDC5 cells. Forced expression of Hif-2 α distinctly inhibited nuclear localization of Cbfb from the cytoplasm (Figure 3B–C) and knockdown of Hif-2 α by shRNA promoted nuclear localization of Cbfb (Figure 3D). Total Cbfb protein levels were not changed by Hif-2 α overexpression or knockdown in chondrocytes (Figure 3B and 3D). Furthermore, Hif-2 α -mediated Runx2 inhibition was rescued by Cbfb transient transfection in chondrocytes (Figure 3E). These results indicate that Hif-2 α enhanced Runx2 polyubiquitination-mediated proteasomal degradation in chondrocytes due to the failure of Cbfb and Runx2 complex formation.

Hif-2 α interferes with the formation of the Runx2/Cbfb complex in chondrocytes

The formation of heterodimeric complexes between Runx2 and Cbfb protects Runx2 from proteasomal degradation in osteoblasts and chondrocytes (Lim et al., 2015; Park et al., 2016). To determine whether Hif-2 α interferes with the formation of Runx2/Cbfb complexes, immunoprecipitation assays were performed by transfecting chondrocytes with HA-Hif-2 α , Flag-Runx2, and Myc-Cbfb. Hif-2 α dose-dependently reduced the formation of the complex between Cbfb and Runx2 (Figure 4A). These results indicate that Hif-2 α -mediated Runx2 inhibition was due to blocking of Cbfb and Runx2 heterodimerization. Since the Runt domain of Runx2 is the binding site of Cbfb, we asked if Hif-2 α could bind to the Runt domain of Runx2 to compete with Cbfb binding to Runx2. Therefore, immunoprecipitation assays were performed on ATDC5 cells by transfecting with HA-Hif-2 α , Flag-Runx2, and Flag- Runt domain deleted Runx2 (Runx2^{delta-Runt}) expression vectors. Interestingly, Hif-2 α bound to full length of Runx2 but not Runx2^{delta-Runt} (Figure 4B). Furthermore, Hif-2 α and Runx2 were mainly co-localized in the nucleus, however, Runx2^{delta-Runt} was localized in the perinuclear area (Figure 4C). Taken together, Hif-2 α bound to the Runt domain of Runx2 directly, which resulted in competing out Cbfb binding to Runx2 and accelerating Runx2 degradation by interruption of Runx2/Cbfb complex formation.

Hif-2 α is a novel inhibitor for chondrocyte maturation through inhibition of Runx2

Based on Hif-2 α inhibition of Cbfb nuclear translocation and Runx2 expression *in vitro*, we investigated whether Runx2 and Cbfb expression was diminished in Hif-2 α

heterozygote mice *in vivo*. As expected, Cbfb nuclear localization was increased by dark spot staining in the nucleus and Runx2 expression was also increased in the *Hif-2a*^{+/-} scapular chondrocytes (Figure 5A). Next, we performed *ex vivo* chondrocyte differentiation assays using mesenchymal cells derived from *WT* and *Hif-2a*^{+/-} limb bud at E11.5. Mesenchymal cells derived from *Hif-2a* heterozygous mice were more rapidly differentiated into hypertrophic chondrocyte than those of wild type mice in a micromass culture system in the Alcian Blue, von Kossa, and immunofluorescence staining (Figure 5B–C). Indeed, heterozygous *Hif-2a* mice slightly accelerated endochondral bone formation of long bone cartilage compared to *WT* mice (Supplementary Figure 2). These results indicate that *Hif-2a* is a novel inhibitor for chondrocyte maturation through inhibition of Runx2.

DISCUSSION

In this study, we uncovered *Hif-2a* as a novel modulator that inhibits chondrocyte maturation by promoting Runx2 proteasome degradation by interfering with the formation of the Runx2/Cbfb complex in chondrocytes (Figure 6).

Notably, our study showed that *Hif-2a* binds to the Runt domain of Runx2 and interferes with the formation of the Runx2/Cbfb complex. Heterodimerization between Runx2 and Cbfb is important for Runx2 stability and transcriptional activity (Kundu et al., 2002; Yoshida et al., 2002). Loss of Cbfb in chondrocytes or osteoblasts induces rapid proteasomal degradation of Runx2, which delays endochondral bone formation and reduces cortical bone thickness (Lim et al., 2015; Park et al., 2016). *Hif-2a* did not regulate Runx2 or Cbfb transcription, but inhibited the Runx2 and Cbfb heterodimerization and Cbfb nuclear translocation in chondrocytes. As a novel binding factor for the Runt domain of Runx2, *Hif-2a* can compete with Cbfb and other transcription factors such as Hdac4 (Vega et al., 2004), Twist-1 (Hinoi et al., 2006), and Sox9 (Zhou et al., 2006) in chondrocytes. Thus, *Hif-2a* may act as a fine-tuner of a variety of physiologically mature hypertrophic chondrocyte functions, including biomineralization, apoptosis, trans-differentiation, and vasculature invasion in the ossification center.

In addition to the pathological function of *Hif-2a* as a catabolic factor associated with both enhanced chondrocyte hypertrophy and apoptosis in the articular chondrocytes (Saito et al., 2010; Yang et al., 2010), this study shows that *Hif-2a* plays as a new negative regulator for chondrocyte maturation in growth plates. Establishing the correct interaction of *Hif-2a* function between articular chondrocytes and growth plate chondrocytes is an important question that requires further investigation. Hypoxia increases VEGF through upregulation of *Hif-1a*, and increased VEGF inhibits osteogenic differentiation but promotes chondrogenic differentiation. This suggests that *Hif-1a* may have the opposite function in regulating osteoblast or chondrocyte activity (Hwang, Noh, Hong, & Lee, 2020). This study also suggests that *Hif-2a* may have various functions in maintaining the homeostasis of articular chondrocytes and chondrocyte maturation of growth plate.

Longitudinal bone growth is controlled by various endocrine, autocrine, and paracrine factors, which eventually leads to growth plate closure with loss of proliferating chondrocytes (Hallett, Ono, & Ono, 2019; Karimian, Chagin, & Savendahl, 2011). Among

transcription factors, Runx2 is a crucial executor that promotes chondrocyte maturation and mineralization to influence longitudinal bone growth. Mmp13, a downstream target of Runx2, is an important factor in chondrocyte hypertrophy and maturation (D'Angelo et al., 2000; Nagai & Aoki, 2002) and in this study, Hif-2 α inhibited Mmp13 expression through Runx2 degradation. We presented the importance of Hif-2 α -Runx2 pathway in chondrocyte maturation in growth plates. Based on the fact that PTHrP inhibits Runx2 expression through inducing PKA signaling pathway and cyclin-D1-mediated proteasome degradation (Li et al., 2004; Zhang et al., 2009), further studies are required to investigate whether the Hif-2 α -Runx2 pathway could affect the PTHrP pathway in chondrocytes.

Collectively, our results show that Hif-2 α is a novel inhibitor for chondrocyte maturation by interfering with the formation of the Runx2/Cbfb β complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in [repository name “JCP-Hif2a-Runx2-Figures” and “JCP-Hif2a-Runx2-Figures-Raw data”] at <https://drive.google.com/drive/folders/15O7G0gX8ypbl9qFUUkizPEd6v3ycJyXC?usp=sharing> and <https://drive.google.com/drive/folders/18UjNmtxL8LjM9fcgU0ovSIN2WBioVYEA?usp=sharing>.

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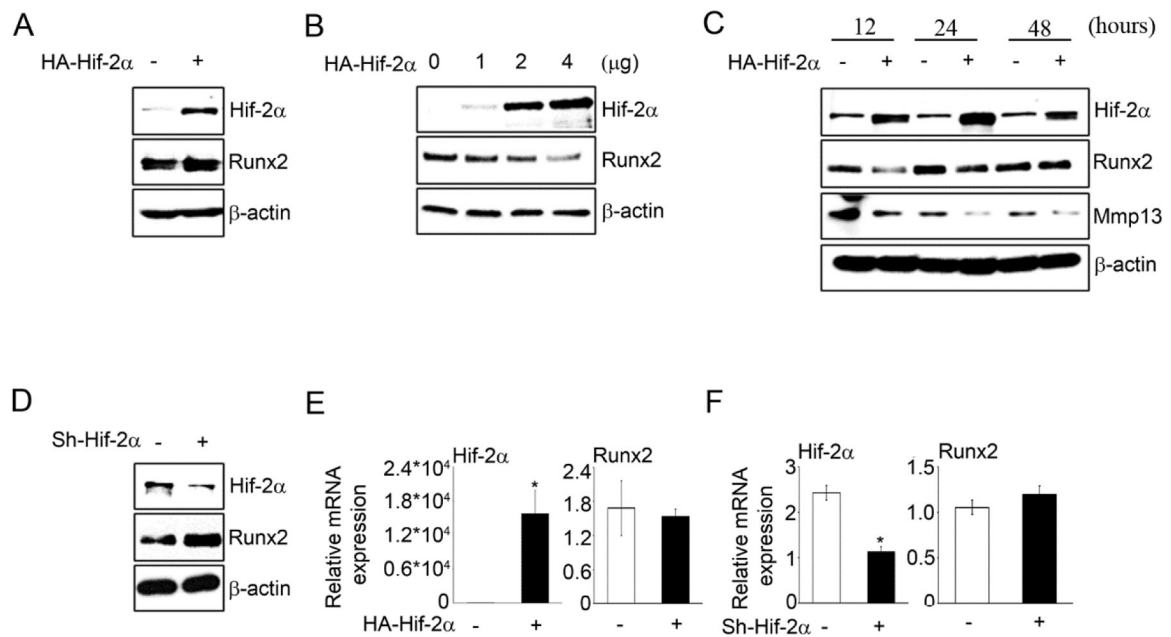


Figure 1. Negative regulation of Hif-2 α on Runx2 expression in chondrocytes

(A) Runx2 protein levels were assessed in ROS17/2.8 osteosarcoma cells after transfection of HA-Hif-2 α for 24 h. (B) Runx2 was evaluated by transfection with HA-Hif-2 α 1, 2, 4 μ g in ATDC5 cells. (C) Runx2 and Mmp13 were assessed in ATDC5 cells time dependently after transfection of HA-Hif-2 α for 12, 24 and 48 h, respectively. (D) Runx2 protein levels were assessed in ATDC5 cells after sh-control-GFP or sh-Hif-2 α -GFP infection. (E-F) Runx2 mRNA expression was determined under transfection of HA-Hif-2 α or infection of after sh-control-GFP or sh-Hif-2 α -GFP. *P < 0.01 vs. control (n=3)

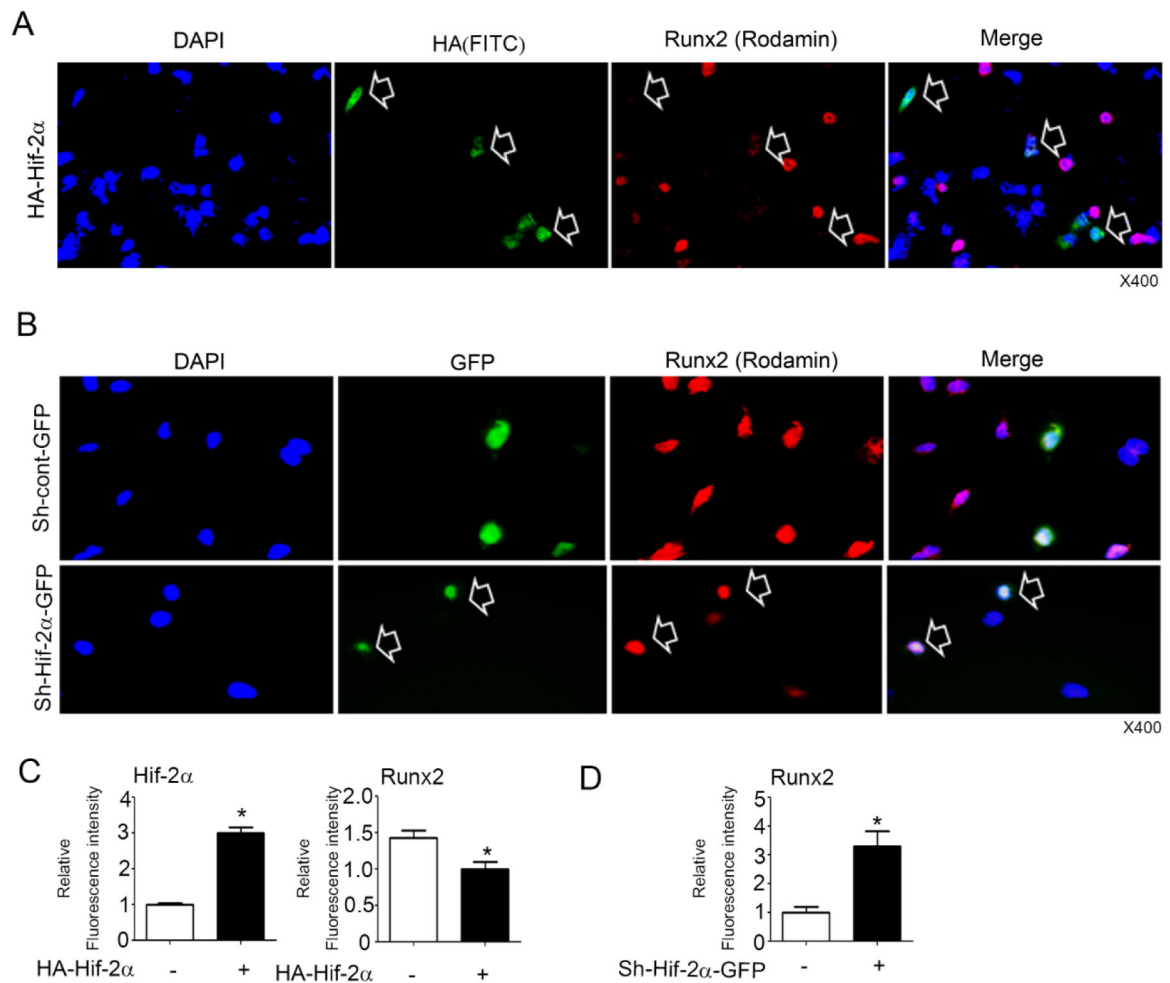


Figure 2. Hif-2 α inhibits endogenous Runx2 level in chondrocytes

(A) Runx2 was determined by immunofluorescence staining after transient transfection with HA-Hif-2 α in ATDC5 cells. (B) Runx2 was determined after infection with sh-control-GFP or sh-Hif-2 α -GFP in ATDC5 cells. Arrow indicates up- and down-regulated Hif-2 α and Runx2 levels in single cells as green and red colors, respectively. Blue is the DAPI staining of the nucleus. X400. (C-D) The fluorescence intensity was analyzed by the Leica and Image J program (n=4–12 for each group).

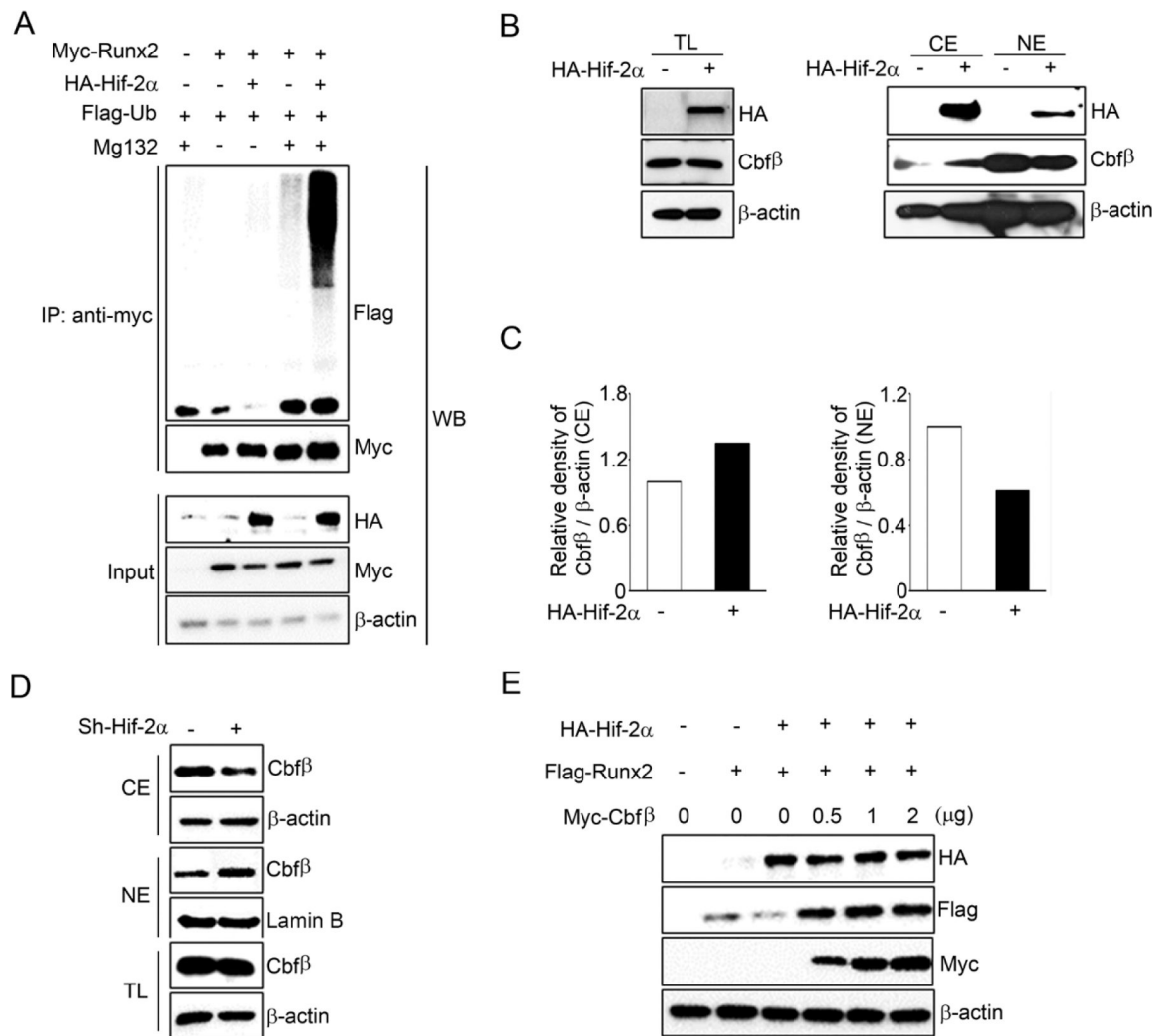


Figure 3. Hif-2 α enhances Runx2 polyubiquitination

(A) Polyubiquitination assay was performed after transfection with expression vectors HA-Hif-2 α , Myc-Runx2, and Flag-Ub in ATDC5 cells. Cells were treated with or without 10 μ M MG132. (B) Expression and subcellular location of Cbf β was determined by western blotting after transfection with HA-Hif-2 α for 24 hours at total lysates (TL), nuclear (NE) and cytoplasmic (CE) extracts. (C) Relative density of Cbf β was normalized with β -actin in CE and NE fractions and analyzed by image J program. (D) ATDC5 cells were infected with sh-Hif-2 α or sh-control lentiviral particles and Cbf β was examined by western blot analysis using TL, NE and CE extracts. (E) Cells were co-transfected with vectors expressing Hif-2 α , Runx2, Cbf β and each protein levels were analyzed by Western blotting with anti-HA, anti-Flag, and anti-Myc antibodies, respectively.

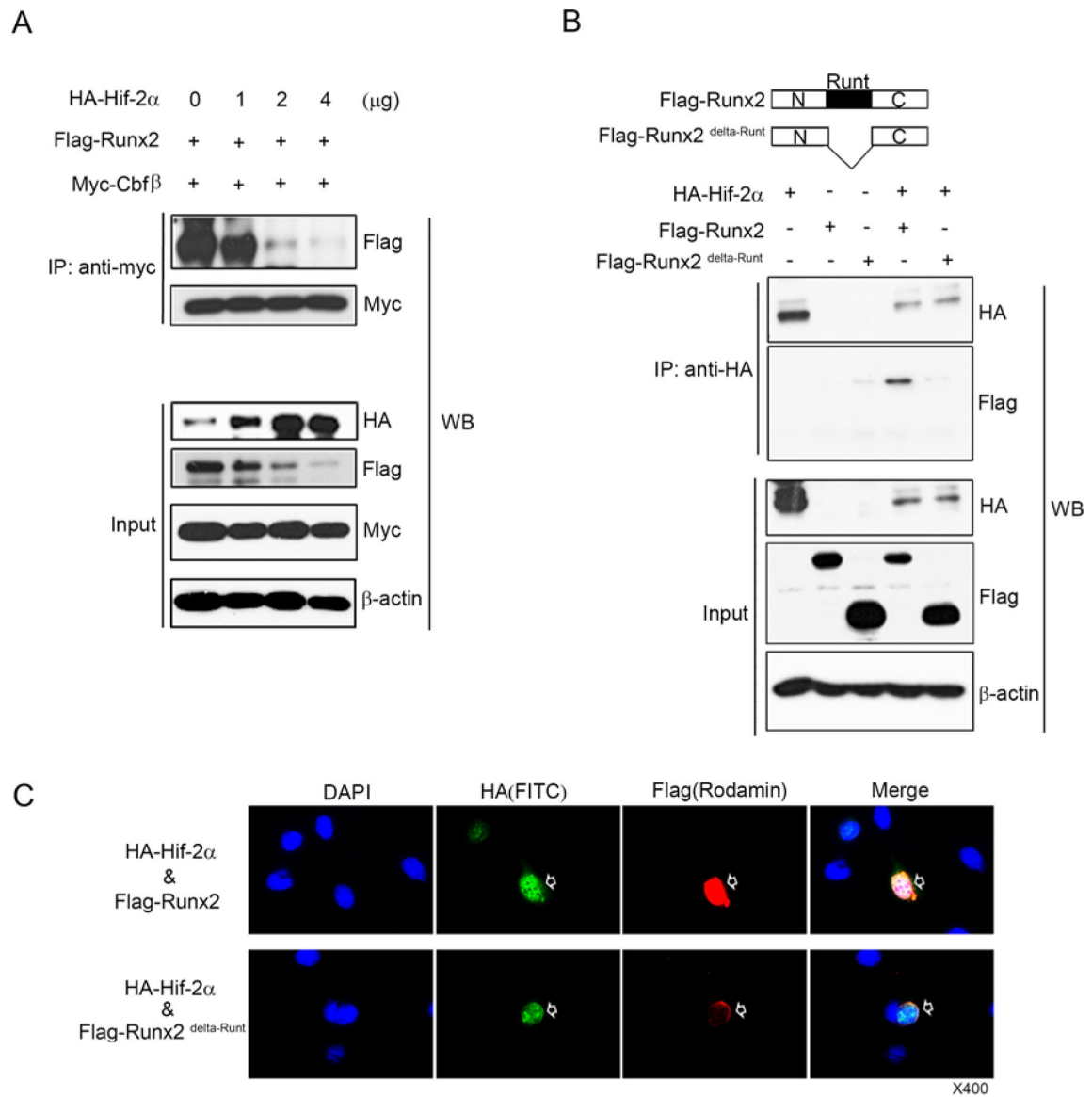


Figure 4. Inhibition of Runx2/Cbf β complex formation by Hif-2 α binding to Runt domain of Runx2

(A) ATDC5 cells were co-transfected with vectors expressing Flag-Runx2, Myc-Cbf β and HA-Hif-2 α . Total extracts were immunoprecipitated with anti-Myc antibody and Runx2 was detected by western blotting (WB) with anti-Flag antibody. (B) ATDC5 cells were co-transfected with Flag-Runx2 and Flag-Runx2^{delta-Runt} expression vectors. Total extracts were immunoprecipitated with anti-HA antibody-tagged bead, and Runx2 and Runx2^{delta-Runt} were detected by western blotting with anti-Flag-HRP antibody. (C) Subcellular localization of Hif-2 α , Flag-Runx2, and Flag-Runx2^{delta-Runt} protein in chondrocytes after co-transfection of HA-Hif-2 α with Flang-Runx2 or Flag-Runx2^{delta-Runt} expression vectors. Hif-2 α and Runx2 were mainly co-localized in the nucleus, however, Runx2^{delta-Runt} was localized in the perinuclear area.

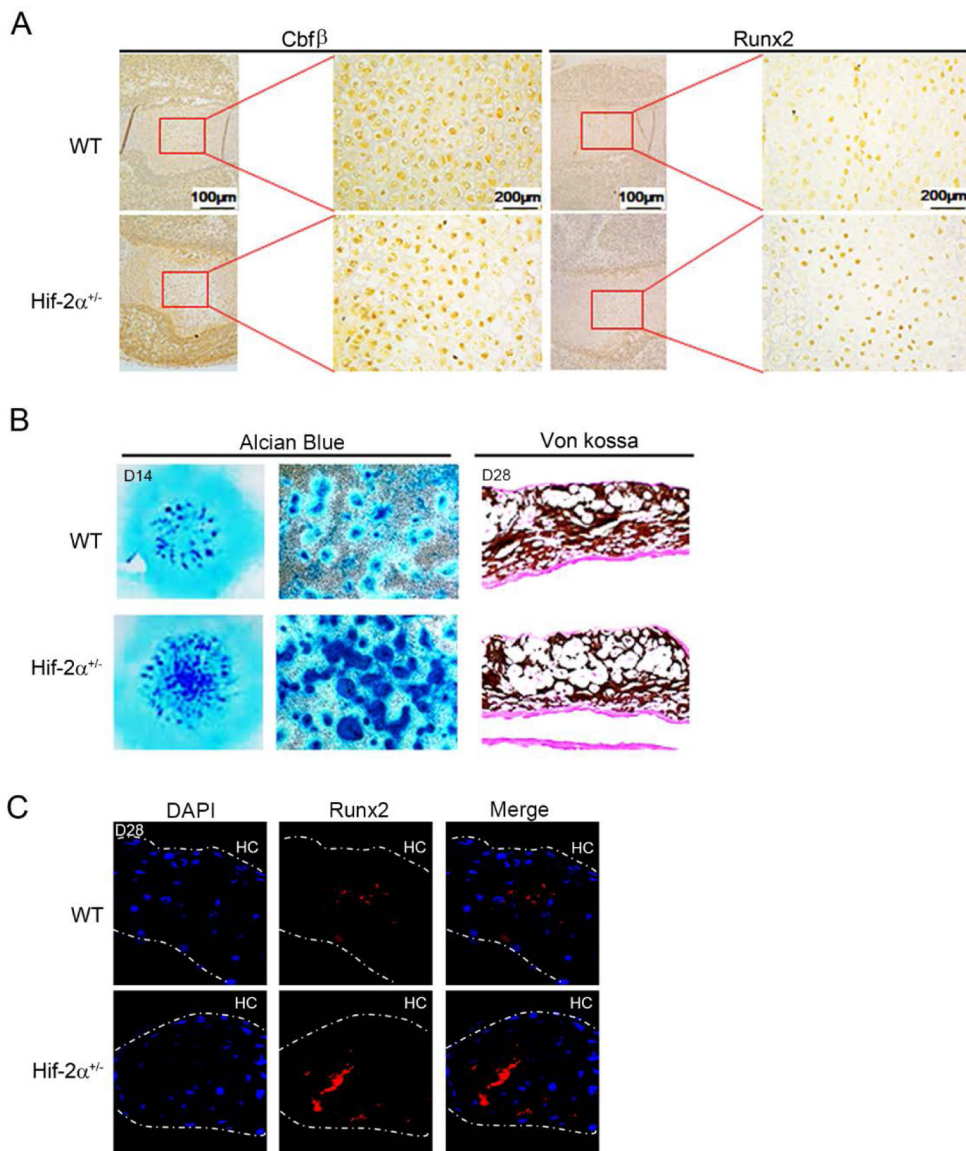


Figure 5. Accelerated chondrocyte differentiation in *Hif-2α^{+/-}* mice

(A) Expression of *Cbfb* and *Runx2* were determined in *Hif-2α^{+/-}* and *WT* scapular chondrocytes at embryonic day 15.0 by immunohistochemistry. (B) For micromass culture, mesenchymal cells were obtained on day 11.5 of embryos from *WT* and *Hif-2α^{+/-}* mice. Chondrocyte differentiation was assessed with Alcian Blue and von Kossa staining. (C) *Runx2* (red) was detected in *Hif-2α^{+/-}* and *WT* micromass cultured cells by immunofluorescence staining. Nucleus was stained by DAPI (blue). HC, hypertrophic chondrocytes. Magnification, x40.

A

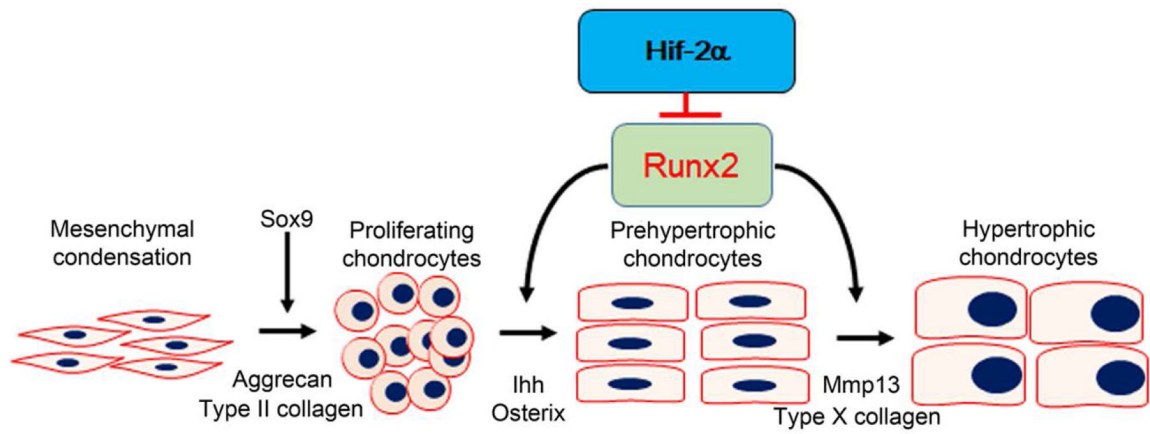


Figure 6. Schema of Hif-2 α as a negative regulator of Runx2 during chondrocyte maturation
 During endochondral ossification, Runx2/Cbfb is necessary for chondrocyte differentiation and maturation in growth plates. Hif-2 α inhibits chondrocyte maturation through interfering Runx2/Cbfb complex formation to degrade Runx2.