HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2023 July 01.

Published in final edited form as:

Bone. 2022 July ; 160: 116418. doi:10.1016/j.bone.2022.116418.

Overexpression of transcription factor FoxA2 in the developing skeleton causes an enlargement of the cartilage hypertrophic zone, but it does not trigger ectopic differentiation in immature chondrocytes

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Abstract

We previously found that FoxA factors are necessary for chondrocyte differentiation. To investigate whether FoxA factors alone are sufficient to drive chondrocyte hypertrophy, we build a FoxA2 transgenic mouse in which FoxA2 cDNA is driven by a reiterated Tetracycline Response Element (TRE) and a minimal CMV promoter. This transgenic line was crossed with a *col2CRE;Rosa26*$^{rtTA/+}$ mouse line to generate *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ mice for inducible expression of FoxA2 in cartilage using doxycycline treatment. Ectopic expression of FoxA2 in the developing skeleton reveals skeletal defects and shorter skeletal elements in E17.5 mice. The chondro-osseous border was frequently mis-shaped in mutant mice, with small islands of col.10+ hypertrophic cells extending in the metaphyseal bone. Even though overexpression of FoxA2 causes an accumulation of hypertrophic chondrocytes, it did not trigger ectopic hypertrophy in the immature chondrocytes. This suggests that FoxA2 may need transcriptional co-factors (such as Runx2), whose expression is restricted to the hypertrophic zone, and absent in the immature chondrocytes. To investigate a potential FoxA2/Runx2 interaction in immature

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Credit authorship contribution statement

NB contributed to Figs. 2 and 3, SB contributed to Figs. 4 and 5, SM contributed to Figs. 6 and 7, EK and ABL contributed to Fig. 2, KH and RP maintained and genotyped the mouse lines used and produced the samples, RK contributed to Fig. 1, RP contributed to Fig. 5, LG and VR data analysis and discussion, AMI study design, data analysis, manuscript writing.

Declaration of competing interest

All authors state that they have no conflicts of interest.

chondrocytes versus hypertrophic cells, we separated these two subpopulations by FACS to obtain $CD24^{+}CD200^{+}$ hypertrophic chondrocytes and $CD24^{+}CD200^{-}$ immature chondrocytes and we ectopically expressed FoxA2 alone or in combination with Runx2 via lentiviral gene delivery. In $CD24^{+}CD200^{+}$ hypertrophic chondrocytes, FoxA2 enhanced the expression of chondrocyte hypertrophic markers *collagen 10*, *MMP13*, and *alkaline phosphatase*. In contrast, in the $CD24^{+}CD200^{-}$ immature chondrocytes, neither FoxA2 nor Runx2 overexpression could induce ectopic expression of hypertrophic markers *MMP13*, *alkaline phosphatase*, or *PTH/PTHrP receptor*. Overall these findings mirror our in vivo data, and suggest that induction of chondrocyte hypertrophy by FoxA2 may require other factors in addition to Runx2 (i.e., Hif2α, MEF2C, or perhaps unknown factors), whose expression/activity is rate-limiting in immature chondrocytes.

Keywords

Cartilage biology; FoxA2; Runx2; Chondrocyte hypertrophy; Endochondral ossification

1. Introduction

Endochondral ossification is an essential process of skeletal development that begins with a series of sequential stages of chondrocyte differentiation and maturation leading to the systematic replacement of the mature cartilage with bone. The cells at the initial stage of differentiation are small round immature chondrocytes that express chondrogenic transcription factors Sox5, Sox6 and Sox9 and an extracellular matrix composed of aggrecan and collagen type II [1]. The immature round cells convert into flattened chondrocytes that proliferate and stack into longitudinal columns, and subsequently transit into prehypertrophic stage and further undergo terminal differentiation into hypertrophic chondrocytes. The prehypertrophic chondrocytes express parathyroid hormone 1 receptor (Pth1R) and Indian hedgehog (Ihh) while the hypertrophic cells express collagen type X (Col10a1) [1]. The process of hypertrophic differentiation of chondrocytes is regulated by distinct families of transcription factors including FoxA(1–3), Runx (1–3), Mef2, and HIF family members [1–11]. Runx2 and Runx3 are expressed in chondrocytes as they initiate differentiation, and loss of these factors (in genetically engineered mice) severely delays or blocks chondrocyte hypertrophy in a number of developing bones [2–4]. Ectopic expression of Runx2 in immature chondrocytes drives premature chondrocyte hypertrophy in some (but not all) skeletal elements and induces expression of collagen X and other hypertrophic markers, both in vivo [6–8] and in vitro [9]. In addition to Runx family members, MEF2C and MEF2D also play a critical role in modulating chondrocyte hypertrophy. They do so either directly, by controlling expression of various differentiation markers (i.e., Indian Hedgehog, PTHrP Receptor, collagen type X) or indirectly, by promoting Runx2 expression [10].

In prior work, we demonstrate that FoxA family members are crucial regulators of the hypertrophic chondrocyte differentiation program [11]. FoxA factors bind to conserved binding sites in the collagen X enhancer and can promote the expression of a collagen X-luciferase reporter in both chondrocytes and fibroblasts. Mice engineered to lack expression of both FoxA2 and FoxA3 in their chondrocytes display defects in chondrocyte hypertrophy,

alkaline phosphatase expression, and mineralization in their sternebrae and in addition exhibit postnatal dwarfism that is coupled to significantly decreased expression of both collagen X and MMP13 in their growth plates [11]. These findings demonstrate that FoxA factors are necessary for chondrocyte differentiation, but do not answer whether FoxA factors alone can drive the differentiation process in cartilage.

To answer this question, we built a FoxA2 transgenic mouse line using a Foxa2 cDNA construct driven by a reiterated Tetracycline Response Element (TRE) and a minimal CMV promoter. This transgenic FoxA2 (*TgFoxA2*$^{+/-}$) mouse line was crossed with a *col2CRE;Rosa26*$^{rtTA/+}$ mouse line to generate *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ embryos for conditional and inducible expression of FoxA2 using doxycycline during skeletal development. Given the importance of FoxA2 for the hypertrophic chondrocyte differentiation program, we hypothesized that FoxA2, if forcefully expressed in the immature cells, can drive their differentiation into hypertrophic chondrocytes. Using various experimental approaches, in the present study we examine the role of FoxA2 in skeletal growth. Here we provide evidence that mis-expression of FoxA2 alone is not sufficient to drive the differentiation of immature into hypertrophic chondrocytes suggesting other hypertrophic factors (absent in the immature chondrocytes) may be needed, or other factors present specifically in the immature chondrocytes could interfere with ectopically FoxA2-driven hypertrophy.

2. Materials and methods

2.1. Generation of TgFoxA2 transgenic mice

A DNA fragment covering the entire coding region of mouse FoxA2 (sequence data available under accession number NM_010446) was cloned into the BamHI/*Cla*I of a MCS (multiple cloning site) for pTRE-Tight expression vector (Clontech cat#631059). The construct was injected into the pronuclei of fertilized eggs from C57Bl/6 mice. Transgenic mice were identified by RT-PCR using the following primers: pTRE Forward: GTG TAC GGT GGG AGG CCT AT and pFoxA2 Reverse: TCA TGT TGC TCA CGG AAG AG. Genotyping was performed by PCR analysis using tail genomic DNA. All animal studies were approved by the Northeastern University Standing Committee on Animals.

2.2. Generation and analysis of col2CRE; Rosa26$^{rtTA/+}$; TgFoxA2$^{+/-}$ triple transgenic mice

The *Col2Cre* deleter strain [12] was kindly provided by Dr. Bjorn Olsen at Harvard School of Dental Medicine. The Rosa26$^{rtTA/+}$; mice were purchased from JAX (cat#006965) and genotyped according to JAX protocols. The *col2Cre* male mice were mated with *Rosa26*$^{rtTA/rtTA}$*; TgFoxA2*$^{+/-}$ females and the pregnant dames were placed at 9.5 dpc on 2mg/ml Doxycycline (Sigma, cat#D9891) in water supplemented with 5% sucrose (VWR, cat#M117). All mice were kept on a mixed outbred background. Alcian Blue-Alizarin Red staining of the *col2CRE; Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ skeletons was performed as previously described [11].

2.3. Histological staining

For histological staining, E16.5-E17.5 embryos were fixed in 4% buffered paraformaldehyde overnight at 4 °C, paraffin-embedded and sectioned at 7 μm, and stained with Hematoxylin/Eosin (Sigma, cat# GHS216). For cartilage glycosaminoglycans visualization, the samples were stained with Alcian Blue 8GX (Sigma, cat#A5268) followed by counterstaining with Nuclear Fast Red (Vector, cat#H03493). In order to visualize alkaline phosphatase activity, the samples were stained with NBT/BCIP (Sigma, cat#11681451001) followed by counterstaining with Methyl Green (Vector, cat#H-3402). Von Kossa staining was performed using a "Von Kossa method for Calcium" kit from Polysciences (cat# 24633-1) according to manufacturer's protocol. TRAP staining was performed using a leukocyte acid phosphatase (TRAP) kit (Sigma-Aldrich, Cat# 387A-1KT) according to the manufacturer's protocol.

2.4. Histomorphometry measurements

Histomorphometry was performed using ImageJ image processing and analysis software (Version 1.6.0_24; Java, National Institute of Health, USA). An average limb length was calculated from N = 3 embryos per genotype. An image analysis application was designed to measure the heights of hypertrophic and non-hypertrophic regions across the humerus of control (*col2CRE;Rosa26*$^{rtTA/+}$) and transgenic mice (*col2CRE;Rosa26*$^{rtTA/+}$*; TgFoxA2*$^{+/-}$). We calibrated the images by using a global scale bar of 100 μm and we measured the corresponding lengths of hypertrophic zone, non-hypertrophic zone, primary ossification center (POC), and whole limb length for each section. For quantification of cell diameter, we measured the diameter of the last three hypertrophic cells closest to the POC. To determine if measurements made by Image J were statistically different, we used the paired Student's *t*-test (two-tailed) and the differences were considered statistically significant at $p < 0.05$.

2.5. Immunohistochemistry

For immunohistochemistry, we used both frozen- and paraffin-embedded sections. The following antibodies were used: FoxA1 (Abcam, cat#ab23738), FoxA2 (Millipore, cat#07-633), FoxA3 (Santa-Cruz, cat#sc-25357), Sox9 (Millipore, cat#AB5535), MMP13 (Abcam, cat#ab39012), Ki67 (Cell Signaling Technology, cat#9129S), col.X (Abcam, cat#ab58632), p57 (Bioworld, cat#BS6876), cleaved caspase-3 (Cell Signaling, Cat#9661S). Antigen retrieval of tissue sections to enhance epitope exposure was performed by Citrate at 65 °C for 1–3 h (p57, Ki67, FoxA1, FoxA2, FoxA3, Sox9) or by 2 mg/ml hyaluronidase at 37 °C for 15 min (MMP13, col.X). The sections were incubated with Dual Endogenous Enzyme Block (Dako, cat#S200380) for 30 min at room temperature in order to suppress endogenous alkaline phosphatase and peroxidase enzymes. Blocking was performed in 0.5% TNB buffer (PerkinElmer, cat#FP1020) for 1 h. The samples were washed with TBST (TBS with 0.05% TWEEN® 20 (Sigma, cat#P1379)) and incubated with anti-rabbit IgG horseradish peroxidase (PerkinElmer, cat#NE-F812001EA), for 45 min at room temperature. After washing the samples in TBST, the slides were incubated for 5 min in a tyramide-biotin working solution, which was prepared in a 1:50 dilution using the TSA™ Biotin System (PerkinElmer cat#NEL700A001KT). For detection, the samples were incubated in strepdavidin conjugated with Alexa Flour 568 dye, diluted 1:500 in 0.5% TNB buffer, for

1 h in the dark at room temperature. Lastly, the samples were mounted using ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen, cat# P36966).

2.6. Flow cytometry and cell sorting of single-cell suspension

Hindlimb growth plates from 1-day old pups were dissected out and the cells were dissociated by enzymatic digestion with collagenase II (0.1%, w/v) and trypsin (0.05%, w/v) at 37 °C in a CO_2 incubator for 2 h, as described previously [13]. The cell suspension was passed through a 70 μm cell strainer, and the cells were resuspended in DMEM containing high glucose (4.5 g/l), 1% L-glutamine, 10% fetal bovine serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. To separate subpopulations of chondrocytes, the cells were stained with the following antibodies: FITC conjugated anti-mouse CD24 (Biolegend Cat#101805) and APC conjugated anti-mouse CD200 (Biolegend Cat#123809) The chondrocyte subpopulations were isolated by fluorescence-activated cell sorting (FACS) to obtain $CD24^+$ $CD200^+$ prehypertrophic/hypertrophic chondrocytes and $CD24^+$ $CD200^-$ immature chondrocytes as described previously [14,15].

2.7. Lentiviral gene transfer

Lentiviruses generated from FoxA2-P2A (CW304886, OriGene Technologies, Inc. Rockville, MD, USA) or FoxA2-P2A-Runx2-P2A (CW304887, OriGene Technologies) synthesis and inserted into pLenti-C-mGFP-P2APuro vector (PS100093, OriGene Technologies) were purchased from OriGene Technologies. The viral particles were provided at a concentration of 10^7 transduction units (TU)/ml and mouse $CD24^+$ $CD200^+$ prehypertrophic/hypertrophic and $CD24^+$ $CD200^-$ immature chondrocytes were transduced at a multiplicity of infection of 10 in serum free medium supplemented with 6 μg/ml polybrene (hexadimethrine bromide) for 24 h. After transduction, the cells were treated with 100 ng/ml BMP2 or proportionate volumes of vehicle (PBS) in complete medium containing 10% fetal bovine serum for 48 h.

2.8. Gene expression analysis

RNA was isolated using an RNeasy Minikit (Qiagen) according to the manufacturer's instructions. A two-step linear amplification protocol was used to amplify the messenger RNA using the Arcturus RiboAmp Plus RNA Amplification Kit (Life Technologies), with 10 ng total RNA as input. Reverse transcription was performed in a 20 μl reaction by iScript Advanced cDNA synthesis kit (Bio-Rad Laboratories, CA, USA) following the protocol supplied by the manufacturer. Quantitative PCR detection of genes was measured using Quantifast SYBR Green QPCR kit (Qiagen) in a total volume of 20 μL. A StepOnePlus Real-Time PCR System (Applied Biosystems, USA) was used for amplification according to the following cycling conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C. PCR product size and the presence of a single amplicon were verified by electrophoresis on 2.5% agarose gels. The Ct values for PPIB gene were used to normalize the expression level of the gene of interest using the ΔΔCt method. Exon-spanning primers for quantitative real-time PCR (qPCR) were designed and used as listed below:

FoxA2-F: CAAACCTCCCTACTCGTACATCTCGC

FoxA2-R: GTAGAAAGGGAAGAGGTCCATGATCCACT

Runx2-F: CCCAGCCACCTTTACCTACA

Runx2-R: TATGGAGTGCTGCTGGTCTG

Aggrecan-F: CACGCTACACCCTGGACTTTG

Aggrecan-R: CCATCTCCTCAGCGAAGCAGT

COL10-F: GCAGCATTACGACCCAAGAT

COL10-R: GCTTCTTTTCCTTGGGGTTC

MMP13-F: ATCCTGGCCACCTTCTTCTT

MMP13-R: TTTCTCGGAGCCTGTCAACT

ALP-F: GCTGATCATTCCCACGTTTT

ALP-R: CTGGGCCTGGTAGTTGTTGT

PTH1R-F: GCTGCTCAAGGAAGTTCTGC

PTH1R-R: GCACATCCTTGTTCTCTTTAGACTC

PPIB-F: TTCTTCATAACCACAGTCAAGAGC

PPIB-R: ACCTTCCGTACCACATCCAT.

2.9. Statistical analysis

Data were expressed as mean ± standard error of the mean (s.e.m.). All data were analyzed using the student's *t*-test. A p-value below 0.05 was considered statistically significant.

3. Results

3.1. FoxA 1–3 are highly expressed in the hypertrophic zone (HZ) of the growth plate (GP) during skeletogenesis

We previously discovered that forkhead box A (FoxA) transcription factors are key regulators of chondrocyte hypertrophy [11]. We followed FoxA 1–3 expression throughout fetal skeletal development and we observed that all FoxA factors (1–3) are highly expressed in the hypertrophic zone (HZ) of the GP in both E15 embryos (Fig. 1b1–3) and newborn P0 mice (Fig. 1c1–3). None of the FoxA factors is expressed in the E12 chondrogenic condensations (Sox9+) that prefigure the future skeletal elements (Fig. 1a1–4). Unlike FoxA3, which is expressed in a broader domain throughout the GP (Fig. 1b3, c3), or FoxA1, which is highly expressed in the HZ but very little elsewhere (Fig. 1b1, c1), FoxA2 is expressed in two discrete domains: the HZ and the RZ (resting zone) in the periarticular region of the growth plate (Fig. 1b2, c2). Finally, none of the FoxA factors are expressed in the metaphyseal bone (Fig. 1c1.1, c2.1, c3.1). The expression of the FoxA factors primarily in hypertrophic chondrocytes is consistent with their role as regulators of cartilage hypertrophy.

3.2. Ectopic expression of FoxA2 in the developing skeleton results in shorter skeletal elements

We generated transgenic mice (*TgFoxA2*$^{+/-}$) containing the FoxA2 cDNA driven by a reiterated Tetracycline Response Element (TRE) (Fig. 2A). The *TgFoxA2*$^{+/-}$ mice were in turn mated to mice containing a reverse tetracycline transactivator (*rtTA*) knocked into the ROSA 26 locus downstream of a "floxed" STOP transcription cassette [16] (Jax, cat#006965) (Fig. 2B). Finally, the resulting progeny *ROSA26*$^{rtTA/+}$*; TgFoxA2*$^{+/-}$ were mated to mice expressing the Cre recombinase driven by the collagen II promoter *col2Cre* mice [12] (Fig. 2B). Administration of tamoxifen to the triple transgenic mice induced *col2Cre* driven recombination and thus expression of the *rtTA*. Subsequent administration of doxycycline in the drinking water induced expression of the FoxA2 transgene throughout the developing cartilage, beyond its normal pattern of expression in the hypertrophic zone (Fig. 2B). FoxA2 is not expressed in proliferating chondrocytes of the control mice, but it becomes expressed in the PZ of the FoxA2 transgenic mice (Fig. 2B, a3 v. b3). FoxA2 is not expressed in the metaphyseal bone (MB) of the control mice, but it becomes expressed in the MB of the FoxA2 transgenic mice, given that (at least some) hypertrophic chondrocytes transdifferentiate in bone cells [17–19] (Fig. 2B, a1 v. b1). FoxA2 is expressed at higher levels in the hypertrophic chondrocytes of the transgenic mice as compared with control littermates (Fig. 2B, a2 v. b2).

Staining of cartilage and mineralized bone using Alcian blue and Alizarin red respectively, revealed skeletal defects in both axial and appendicular skeletons of E17.5 embryos overexpressing FoxA2 in their developing cartilages (Fig. 2C). The overall size of the skeletal elements is decreased, with noticeable differences observed in primary ossification center. The Alizarin Red stained osseous matrix is significantly reduced in hindlimbs and sterna of *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ triple transgenic mice, as compared with control littermates (Fig. 2C).

3.3. Ectopic expression of FoxA2 in the developing skeleton contributes to the expansion of the hypertrophic zone at the expense of the POC formation

In light of the skeletal abnormalities observed in the *col2CRE; Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ triple transgenic mice, we next characterized their cartilage and bone development. During murine skeletal development, osteogenesis sprouting in the mid-diaphyseal hypertrophic cartilage is supported by both osteoblast precursors, invading from the perichondrium [20], as well as by hypertrophic chondrocytes, trans-differentiating to support the bone lineage [17,18,21]. In *col2CRE; Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ triple transgenic mice, the primary ossification center (POC) formation is not delayed; the POC is fully formed at E16.5 (Fig. 3A) and continues to expand by E17.5 (Fig. 3B).

The overall length of limbs is mildly reduced (13%) in the transgenic FoxA2 mice compared to control littermates (Fig. 3E, e1). This is the result of a compensation between a significant reduction of the primary ossification center (POC) area and an enlargement of the hypertrophic zone in the transgenic mice. Histomorphometric measurements reveal that the POC length of *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ transgenic mice is reduced 46% compared with control littermates (Fig. 3E, e2), while the hypertrophic zone shows a 39%

increase in mutant mice (Fig. 3E, e3). Consistently, histological analysis showed extended hypertrophic zones in the hindlimbs (Fig. 3A, B), forelimbs (Fig. 3D) and sterna (Fig. 3C) of *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ mice.

We measured the diameter of the hypertrophic chondrocytes in control and mutant mice, and we did not find any statistical difference, suggesting that enlargement of the hypertrophic zone may be due to an increased number of hypertrophic chondrocytes rather than an increase in cellular volume (Fig. 3E, e5). This accumulation of hypertrophic chondrocytes may come from an increased differentiation of the immature chondrocytes into hypertrophic cells, or from a failure of the hypertrophic chondrocytes to further contribute to the bone lineage. We observed a small (12%) reduction in the length of the non-hypertrophic zone (Fig. 3E, e6), but no difference in the proliferation rate of the immature chondrocytes, as shown by ki67 immunohistochemistry (Fig. 5A, a3–4). This suggests that although FoxA2 overexpression does not affect epiphyseal chondrocyte proliferation, it slightly boosts the differentiation of immature chondrocytes into hypertrophic chondrocytes.

However, the most prominent effect of FoxA2 mis-expression in the developing skeleton is an extension of the hypertrophic chondrocyte fate, at the expense of the primary ossification center (POC) formation. The chondro-osseous border was frequently misshaped in FoxA2 transgenic mice, with small islands of hypertrophic cells extending in the metaphyseal bone, suggesting that chondrocyte hypertrophy was not properly coordinated with osteogenesis (Fig. 3B, b1–2, and D, d1–2). This accumulation of hypertrophic chondrocytes in the FoxA2 transgenic mice may relay a failure or delay of the hypertrophic chondrocytes to further trans-differentiate into bone cells. To further characterize hypertrophy into osteogenesis transition in the mutant mice, we assayed both alkaline phosphatase activity (Fig. 4a3–4) and matrix mineralization by von Kossa staining (Fig. 4a1–2). The mid-diaphyseal bone collar was fully formed, with strong mineralization present in the periosteal cells lining the hindlimbs of both mutant and control mice (Fig. 4a1–2, a3–4). However, the cancellous bone in the FoxA2 transgenic mice displayed little or no trabeculae and less matrix mineralization, as shown by attenuated von Kossa staining, relative to their control littermates (Fig. 4a1–2). In addition, we observed “islands” of col.10+ hypertrophic chondrocytes found occasionally in the metaphyseal bone of the transgenic FoxA2 mice (Figs. 3B, b2, D, d2, 5C, c2). To determine whether this delay in cartilage degradation may be due to defective MMP13 production, we performed immunohistochemistry for MMP13. We did not observe a significant change in MMP13 expression on epiphyseal cartilage from control and mutant mice (Fig. 4a5–6). However, degradation of the hypertrophic cartilage is a complex process involving other metalloproteinases (such as MMP2 and MMP9), in addition to MMP13, as well as various tissue inhibitors of metalloproteinases (TIMPs) [22–24]. As such, it is possible that other factors (MMPs, TIMPs) are differentially regulated in FoxA2 transgenic mice. Finally, we did not see any differences in TRAP+ osteoclasts between control and transgenic FoxA2 mice (Fig. 4a7–8).

3.4. Chondrocyte-specific overexpression of FoxA2 causes an accumulation of hypertrophic chondrocytes, but it cannot trigger ectopic hypertrophy in immature chondrocytes, even in the presence of Runx2

Overall we did not see any differences in proliferation rate (assayed by ki67 expression) (Fig. 5A, a3–4) and matrix proteoglycan deposition (assayed by Alcian Blue staining) (Fig. 5A, a1–2) in immature chondrocytes from FoxA2 transgenic mice versus control littermates. As such, the most prominent feature of the FoxA2 overexpression in the developing skeleton remains expansion of the hypertrophic zone.

To further characterize this enlarged population of hypertrophic cells in the mutant mice, we performed immunohistochemistry for collagen 10, p57 and cleaved caspase-3 expression. We observed an extension of collagen 10 expression in the forelimbs (Fig. 5C, c1–2), hindlimbs (Fig. 5C, c3–4) and sterna (Fig. 5C, c5–6) of *col2CRE;Rosa26*$^{rtTA/+}$*; TgFoxA2*$^{+/-}$ transgenic mice. Similarly, p57 expression domain was expanded in FoxA2 transgenic mice (Fig. 5B, b1–2) proportional with expansion of the hypertrophic zone. This was further supported by a decrease in the expression of pro-apoptotic marker cleaved caspase-3 in the hypertrophic zone/metaphyseal bone, but not in the epithelial tissue, of FoxA2 transgenic mice (Fig. 5B, b3–4, i & ii).

The residual hypertrophic cells outspreading in the metaphyseal bone were also positive for collagen 10 expression (Fig. 5C, c2). The osteoid matrix does not express aberrant collagen 10, despite displaying a delay in cartilage degradation. Interestingly, we also did not see any ectopic collagen 10 expression in the immature epiphyseal chondrocytes, despite extensive FoxA2 overexpression in the skeleton (Fig. 2B). Although we previously showed that FoxA2 is necessary for both collagen 10 and MMP13 expression in chondrocytes [11], FoxA2 does not seem sufficient to trigger spontaneous hypertrophy in the immature chondrocytes. Neither forelimbs, nor hindlimbs or sterna/ribs of the mutant mice display collagen 10 expression outside its established domain, the hypertrophic zone of the epiphyseal cartilage (Fig. 5C). Similarly, MMP13 expression is restricted to late hypertrophic chondrocyte and bone cells, and there is no ectopic expression in immature chondrocytes (Fig. 4a5–6).

This suggests that FoxA2 alone is not enough to drive immature chondrocytes to hypertrophy. FoxA2 may need transcriptional co-factors, whose expression is restricted to the hypertrophic zone, and absent in the immature chondrocytes. One of these potential partners is transcription factor Runx2, a known regulator of both cartilage hypertrophy and osteogenesis [7,25]. To investigate a potential FoxA2/Runx2 interaction in immature versus hypertrophic cells, we separated these two chondrocyte subpopulations by fluorescence-activated cell sorting (FACS) to obtain $CD24^{+}$ $CD200^{+}$ hypertrophic chondrocytes (HC) and $CD24^{+}CD200^{-}$ immature chondrocytes (IC) as described previously (Fig. 6A) [14,15]. As expected, $CD24^{+}CD200^{+}$ hypertrophic chondrocytes express significantly higher levels of *collagen 10*, *FoxA2*, *PTH/PTHrP receptor (PTH1R)* and *Runx2* and much lower levels of *Aggrecan* than $CD24^{+}$ $CD200^{-}$ immature chondrocytes (Fig. 6B).

To investigate a potential FoxA2/Runx2 interaction in immature versus hypertrophic cells, we ectopically expressed FoxA2 alone or in combination with Runx2 via lentiviral gene delivery (Fig. 7). We transduced growth plate chondrocytes with a lentivirus encoding

either control GFP (Lenti-GFP), FoxA2 alone (Lenti-FoxA2-GFP) or FoxA2 plus Runx2 (Lenti-FoxA2-Runx2-GFP) using a P2A peptide to co-express multiple genes at comparable levels under the control of a single CMV promoter. One advantage is that the downstream ORF can be expressed at levels equivalent to that of upstream ORF [26,27]. Consequently, we observed qualitatively similar GFP fluorescence irrespective of where GFP is placed in the lentiviral gene cassette (Fig. 7A). Expression of lentiviral-encoded FoxA2 in chondrocytes increased FoxA2 expression levels 151-fold over the endogenous FoxA2, whereas expression of lentiviral-encoded Runx2 increased Runx2 levels 90-fold (Fig. 7A). In $CD24^{+}CD200^{+}$ hypertrophic chondrocytes, lentiviral FoxA2 enhanced the expression level of chondrocyte hypertrophic markers *collagen 10* (3-fold), *MMP13* (4.8-fold) and *alkaline phosphatase (ALP)* (1.6-fold), while constitutive expression of both FoxA2 and Runx2 by lentiviral transduction boosted these increases significantly (Fig. 7B). In contrast, in $CD24^{+}CD200^{-}$ immature chondrocytes, neither FoxA2 nor Runx2 overexpression could induce ectopic expression of hypertrophic markers *MMP13*, *alkaline phosphatase (ALP)*, or *PTH/PTHrP receptor (PTH1R)* (Fig. 7B). Although we did see an increase in *collagen 10* in response to FoxA2 overexpression in $CD24^{+}CD200^{-}$ chondrocytes, the overall level of expression is reduced in immature chondrocytes, as compared to hypertrophic chondrocytes. Overall these findings mirror our in vivo data, and suggests that induction of chondrocyte hypertrophy by FoxA2 may require other factors in addition to Runx2 (i.e., Hif2α, MEF2C, or perhaps unknown factors), whose expression/activity is rate-limiting in immature chondrocytes.

4. Discussion

Our prior work has shown that mice with conditional deletion of both FoxA2 and FoxA3 in their chondrocytes display defects in chondrocyte hypertrophy, alkaline phosphatase expression, and mineralization in their skeletal elements during fetal and postnatal development [11]. Our current findings show that FoxA2 overexpression leads to an enlargement of the hypertrophic zone, accompanied by a significant reduction of the osteogenic domain. Several events may cause this expansion of the hypertrophic chondrocytes.

A small contribution may come from differentiation of the immature chondrocytes into hypertrophic cells. Even though FoxA2 over-expression did not affect chondrocyte proliferation, it caused a small reduction of the non-hypertrophic zone, which may suggest a faster rate of differentiation pre-hypertrophic cells into hypertrophic chondrocytes.

Another possible explanation for the expansion of the hypertrophic zone could be due to overexpression of FoxA2 in the hypertrophic cells. In *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ mice, expression of the CRE recombinase in the col2+ cells will remove the STOP codon from the Rosa26 locus and allow permanent expression of rtTA in col2+ cells as well as their progeny. As long as the mice receive Doxycycline, all col2+ cells and their descendants will express the FoxA2 transgene. Since col2+ chondrocytes are differentiating into hypertrophic chondrocytes, hypertrophic chondrocytes will express the FoxA2 transgene, as descendants of the col2+ cells. We demonstrate in Fig. 7 that lentiviral overexpression of FoxA2 in hypertrophic chondrocytes generates significant increases in the expression of hypertrophic

markers (*col10*, *ALP*, *MMP13*). As such, the in vivo expansion of the hypertrophic zone could be due to overexpression of FoxA2 in the hypertrophic cells.

Accumulation of hypertrophic chondrocytes could also come from a failure of hypertrophic chondrocytes to further trans-differentiate into osteoblasts. Murine primary ossification center (POC) is formed around E15.5, when the first Osx-expressing osteoblast precursors appear in the perichondrium surrounding the mid-diaphyseal hypertrophic cartilage [20]. The arrival of the osteoblast precursors into the POC is accompanied by blood vessel invasion and further differentiation of precursor cells into bone-forming trabecular osteoblasts [20]. In addition, hypertrophic chondrocytes also contribute to the osteogenic lineage [17–19,21], with some hypertrophic chondrocytes trans-differentiating into osteoblasts [21], while others de-differentiate into a more primordial stem cell phenotype [19,28] and subsequently further differentiate into bone cells. Accumulation of hypertrophic cells in the FoxA2+ transgenic mice, concomitant with reduction of the osteogenic domain, may reflect an impairment of the hypertrophic chondrocytes to further contribute and support the osteogenic lineage.

Another possible cause for the diminished osteogenic domain would be the mis-expression of FoxA2 in osteoprogenitors and osteoblasts. In the current work, we demonstrate that, in wild-type mice, there is no expression of FoxA factors in the bone spongiosa during fetal development. However, in the *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ triple transgenic mice, all collagen 2 expressing chondrocytes and their progeny (hypertrophic chondrocytes and osteoprogenitors) express high levels of FoxA2. Ye et al. previously showed that forced expression of FoxA2 in bone marrow -derived mesenchymal stem cells (BMSC) slowed down osteogenic differentiation in vitro [29]. As such, in vivo mis-expression of FoxA2 in osteoprogenitor cells, in places where it is not normally expressed, may cause a delay in osteogenesis and may explain why FoxA2 transgenic mice have little or no bone trabeculae and less matrix mineralization, relative to their control littermates. Altogether, these findings may also explain why hypertrophic chondrocytes transiting in bone spongiosa downregulate FoxA2 expression, as high levels of FoxA2 would impair osteogenesis [29].

Finally, overexpression of FoxA2 may be interfering with the apoptosis of the hypertrophic chondrocytes, which may cause an enlargement of the hypertrophic region. Presence of pro-apoptotic caspases (−3, −6, −7, −8, −9) was shown within the growth plate of mouse forelimbs at E18 [30]. For all these caspases, there was a gradient increase in activation toward the ossification zone [30]. Immunohistochemistry for cleaved caspase 3 shows a significant loss of pro-apoptotic staining in the hypertrophic zone and in the metaphyseal bone of the transgenic mice, as compared with control mice. The cleaved caspase 3 staining in the neighboring epithelial tissue was unchanged. This is consistent with previously published reports regarding the role of FoxA2 in preventing apoptosis in hepatocytes, colon cancer cells and hepatocellular carcinoma cells [31–33]. In the hypertrophic chondrocytes, FoxA2 overexpression may be decreasing apoptosis, causing an expansion of the hypertrophic region. It is possible that ectopic expression of FoxA2 in the immature cells changes the properties of the hypertrophic chondrocytes (derived from these FoxA2+ overexpressing immature chondrocytes), thereby preventing their elimination

by apoptosis, or transdifferentiation into osteoblasts. Future studies will be needed to address these complex issues.

Altogether, our results demonstrate that FoxA2 is necessary but not sufficient to drive cartilage hypertrophy in the developing skeleton. Our current findings show that extensive FoxA2 overexpression in the developing skeleton is not enough to trigger ectopic hypertrophy in the immature chondrocytes. Despite an enlargement of the hypertrophic zone and an accumulation of collagen 10+ hypertrophic cells, we do not see collagen 10, ALP or MMP13 expression in the non-hypertrophic zone, outside their established domain (hypertrophic cartilage). In contrast, misexpression of Runx2, another transcriptional regulator of chondrocyte differentiation [25,34] causes precocious cartilage hypertrophy and accelerated osteogenesis, in some but not all skeletal elements, in locations where it normally never occurs [6,7]. Conversely, FoxA2/3 alone are not enough to control this process, since despite extensive FoxA2 over-expression in the non-hypertrophic chondrocytes, those are unable to spontaneously activate chondrocyte hypertrophy. One possible explanation could be the differential expression patterns for FoxA factors as opposed to Runx1–3 family. Runx2 and Runx3 are highly expressed in hypertrophic chondrocytes and bone, but not in the immature epiphyseal cells. Runx1 expression is restricted to the developing sternal bars, but very little elsewhere [5,7]. In contrast, we see FoxA2/3 expression extending beyond the hypertrophic zone, in the immature chondrocytes, yet those cells do not express collagen 10 or MMP13. In the appendicular skeleton, FoxA3 is expressed in a broader domain throughout the tibial GP, FoxA1 is highly expressed in the HZ but very little elsewhere, and FoxA2 is expressed in two discrete domains: the HZ and the periarticular region. As a result, if FoxA2/Runx2 work together to regulate chondrocyte hypertrophy, then upon Runx2 overexpression in immature chondrocytes, FoxA2/3 would be available to support Runx2-driven spontaneous hypertrophy in the tibial non-hypertrophic cartilage. However, in mice overexpressing Runx2 in col.2+ cells, only the chondrocostal and tracheal cartilage displayed advanced mineralization, not the appendicular skeleton [7].

We previously published that 6 FoxA binding sites are present within the conserved region of the human collagen X enhancer that are also present upstream of the murine and bovine collagen X genes [11]. These FoxA binding sites flank Runx2 binding sites that are also conserved in the human, murine and bovine collagen X enhancers [11]. Co-transfection of a collagen X luciferase reporter (mcol10-4.6kb-Luc) driven by the mouse collagen X enhancer together with either murine FoxA1, FoxA2, or FoxA3 revealed that the enhancer activity of this regulatory region is significantly boosted by exogenous FoxA factors [11]. Thus, the presence of conserved FoxA and Runx2 binding sites in both mammalian and avian collagen X enhancers suggests that these transcription factors may co-operate to activate the expression of collagen X in hypertrophic chondrocytes of both mammals and birds.

To further investigate whether Foxa2 and Runx2 are sufficient to trigger spontaneous hypertrophy in non-hypertrophic chondrocytes, we compared overexpression of FoxA2 alone versus FoxA2 together with Runx2 in immature chondrocytes (CD24+CD200-) and in hypertrophic chondrocytes (CD24+CD200+). Combined expression of FoxA2 together with Runx2 enhanced the level of chondrocyte hypertrophy markers in $CD24^{+}CD200^{+}$ hypertrophic chondrocytes, but not in immature $CD24^{+}CD200^{-}$ chondrocytes. We did see

an increase in *collagen 10* expression in response to FoxA2 overexpression, however this may reflect differences between in vitro studies (using isolated cells cultured in monolayer) and in vivo studies (subject to 3D regulation of gene expression via dense packaging of cells, encapsulation within a condensed extracellular matrix, and perichondrial signaling). Altogether these results are consistent with the lack of ectopic differentiation in immature chondrocytes in the growth plates of *col2CRE;Rosa26*$^{rtTA/+}$*; TgFoxA2*$^{+/-}$ triple transgenic mice.

Finally, the inability of FoxA2 and Runx2 to induce hypertrophic markers in immature cells might also be due to epigenetic factors (e.g. closed chromatin) or due to the fact that immature chondrocytes express specific factors that inhibit chondrocyte maturation. Recent studies have identified epigenetic processes, such as histone acetylation and methylation, which contribute to the regulation of differentiation and maturation of mesenchymal progenitors, chondrocytes, and osteoblasts [35–40]. It was previously published that SOX9 represses *Col.10* expression in immature/proliferating chondrocytes of the growth plate, so that its expression is restricted to hypertrophic chondrocytes [41]. Discrimination of this concomitant opposing transcriptional control may involve cooperation between SOX9 and different partners such as GLI factors (effectors of hedgehog signaling) [42].

5. Conclusions

In summary, our findings demonstrate that overexpression of FoxA2 in chondrocytes induces an enlargement of hypertrophic zone of the cartilage, but cannot promote the ectopic differentiation of the non-hypertrophic cartilage cells. Ectopic expression of FoxA2 in the immature chondrocytes cannot trigger chondrocyte hypertrophy even in the presence of Runx2. This suggests that: 1) other hypertrophic chondrocytes-specific known factors (such as Mef2C and Hif2a), or unknown factors, may be required for the cooperative interaction between FoxA2 and Runx2 and/or 2) unknown factors within the immature cells may suppress the FoxA2/Runx2 activity and block induction of ectopic hypertrophy. Future studies are needed to investigate the plausible mechanisms in the immature and hypertrophic cells for understanding their role in regulating the effects of FoxA2 in a cell type-specific manner.

Funding

This work was supported by a NIAMS/NIH grant to A.M.I. (1R01AR069671-01A1).

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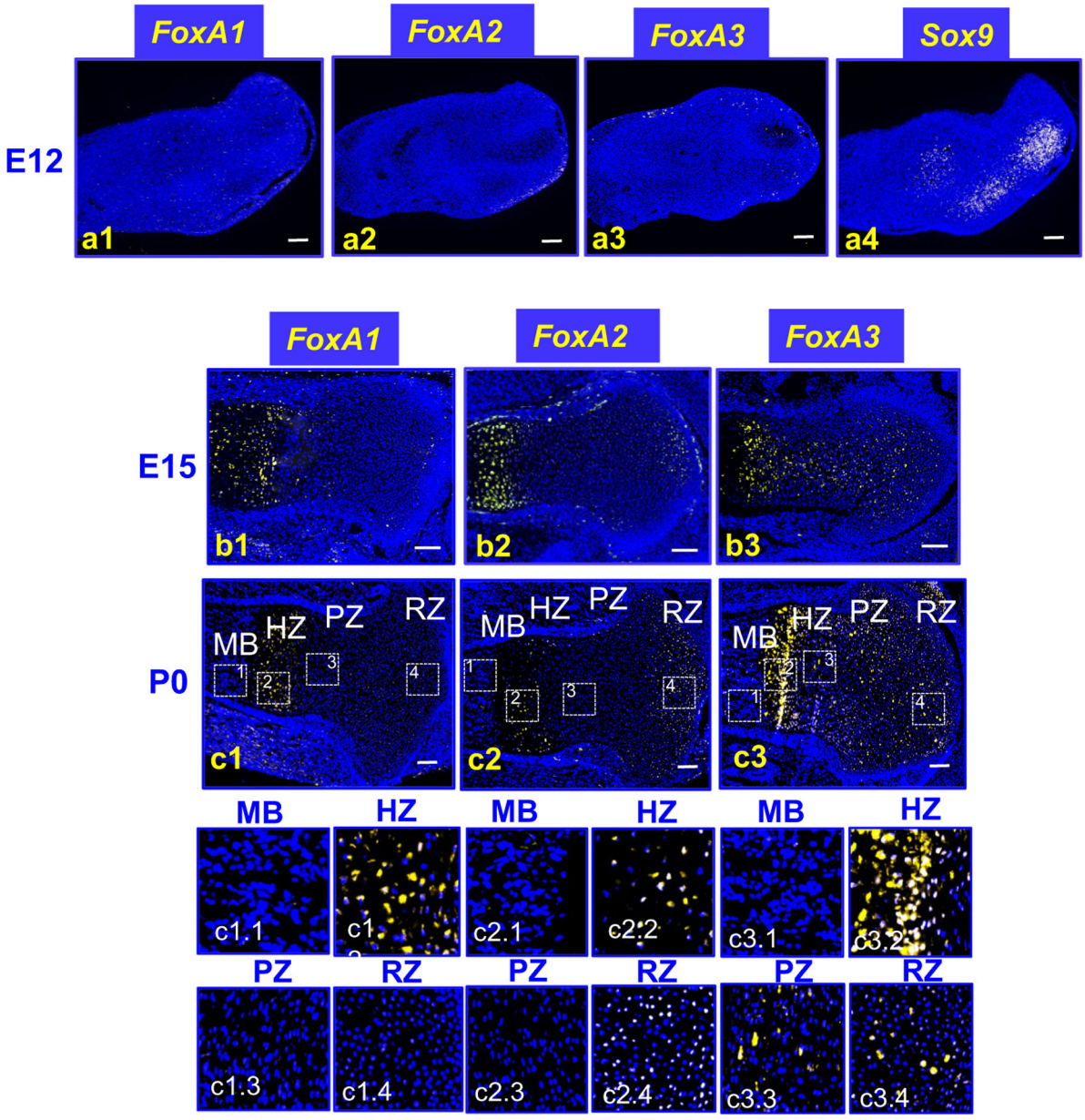

Fig. 1.

FoxA 1–3 are highly expressed in hypertrophic chondrocytes during skeletogenesis. Immunohistochemistry for FoxA1–3 and Sox9 on E12 limb buds (a1–4). Immunohistochemistry for FoxA1–3 on E15 limbs (b1–3). Immunohistochemistry for FoxA1–3 on P0 limbs (c1–3). Counterstain with Hoechst dye (blue), FoxA2 signal (yellow). MB = metaphyseal bone, HZ = hypertrophic zone, PZ = proliferating zone, RZ = resting zone. Representative details, from each zone, are shown in numbered insets (c1(1–4), c2(1–4), c3(1–4)). Scale bars, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

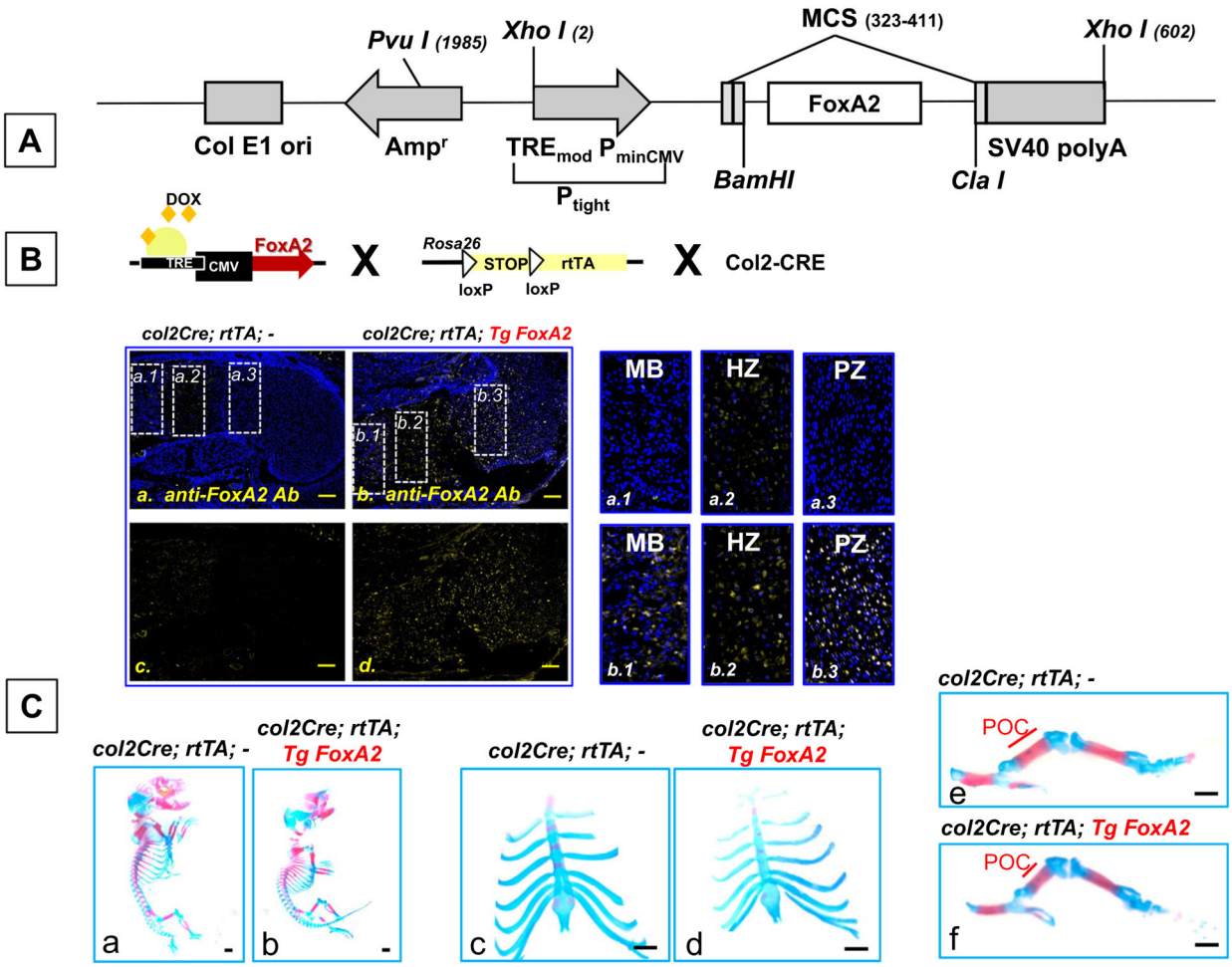

Fig. 2.

Ectopic expression of FoxA2 in the developing skeleton results in shorter skeletal elements. DNA construct map for transgenic mice (*TgFoxA2*) containing the FoxA2 cDNA driven by a reiterated Tetracycline Response Element (TRE) and a minimal CMV promoter. Restriction sites for BamHI and *Cla*I were used for insertion of FoxA2 cdna in the MCS (Multiple Cloning Site) (A). Immunohistochemistry (IHC) for FoxA2 expression in the developing cartilage of control *col2CRE;Rosa26*$^{rtTA/+}$ mice (a, c), or transgenic *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ (b, d) counterstained with Hoechst dye (blue) (a, b) or no Hoechst dye (c, d). Images for FoxA2 IHC (yellow) were taken with the same 1/30s exposure time for both control and transgenic mice. MB = metaphyseal bone, HZ = hypertrophic zone, PZ = proliferating zone. Representative details, from each zone, are shown in numbered insets (a(1–3), b(1–3)). Scale bars, 100 μm. (B). Alcian Blue/ Alizarin Red staining of whole body (a, b), sternum (c, d) and hindlimbs (e, f) of control *col2CRE;Rosa26*$^{rtTA/+}$ mice (a, c, e), and transgenic *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ mice (b, d, f) Scale bars, 1 mm. (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

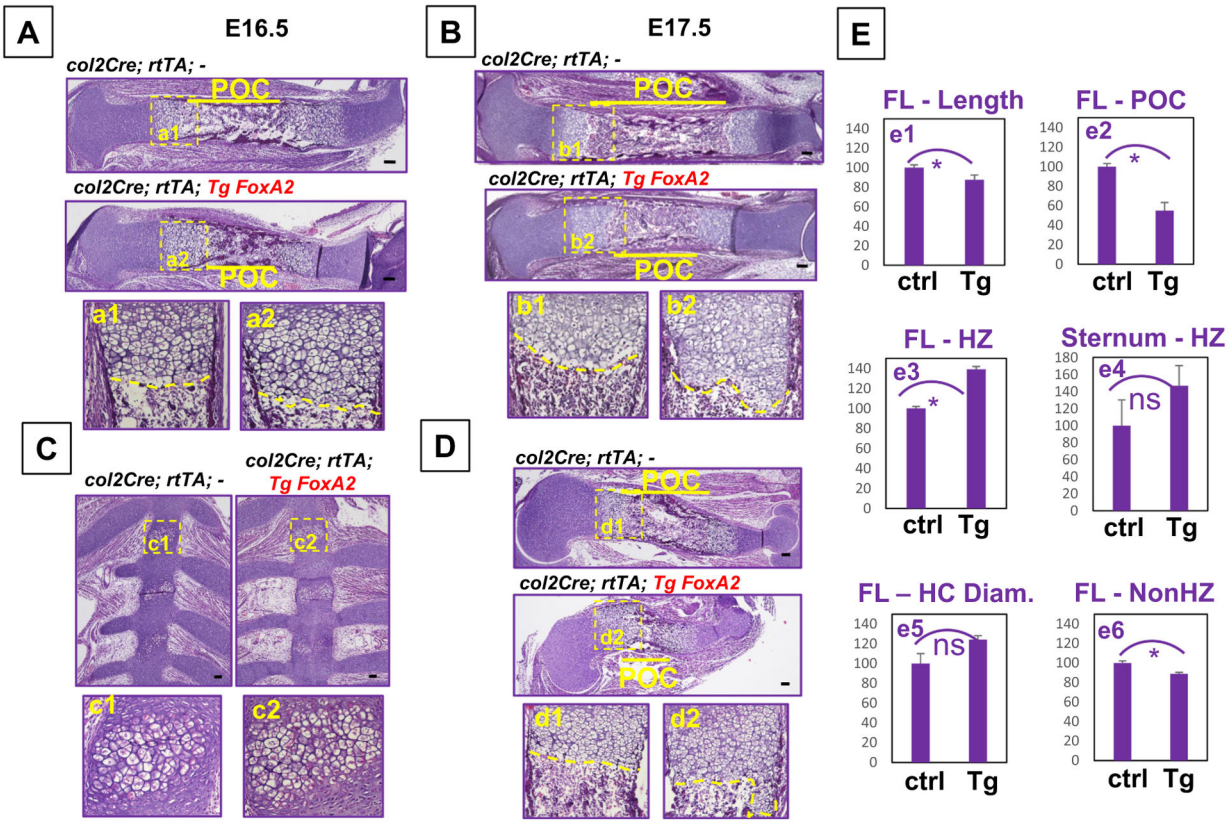

Fig. 3.

Ectopic expression of FoxA2 in the developing skeleton contributes to the expansion of the hypertrophic zone at the expense of the POC (primary ossification center) formation. Histological analysis of E16.5 hindlimbs (A), E17.5 hindlimbs (B), sterna (C) and forelimbs (D) from control *col2CRE;Rosa26*$^{rtTA/+}$ mice (a1, b1, c1, d1) and transgenic *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ mice (a2, b2, c2, d2). Scale bars, 100 μm (A–D). Histomorphometric measurements of control (ctrl) and transgenic (Tg) mice: total forelimb length (e1), primary ossification center (POC) height (e2), hypertrophic zone (HZ) height (e3), sternum hypertrophic zone area (e4), hypertrophic chondrocyte diameter (e5), nonhypertrophic zone height (e6), N = 3 embryos per genotype. Statistical significance by *t*-test is indicated with * denoting $p < 0.05$ (E).

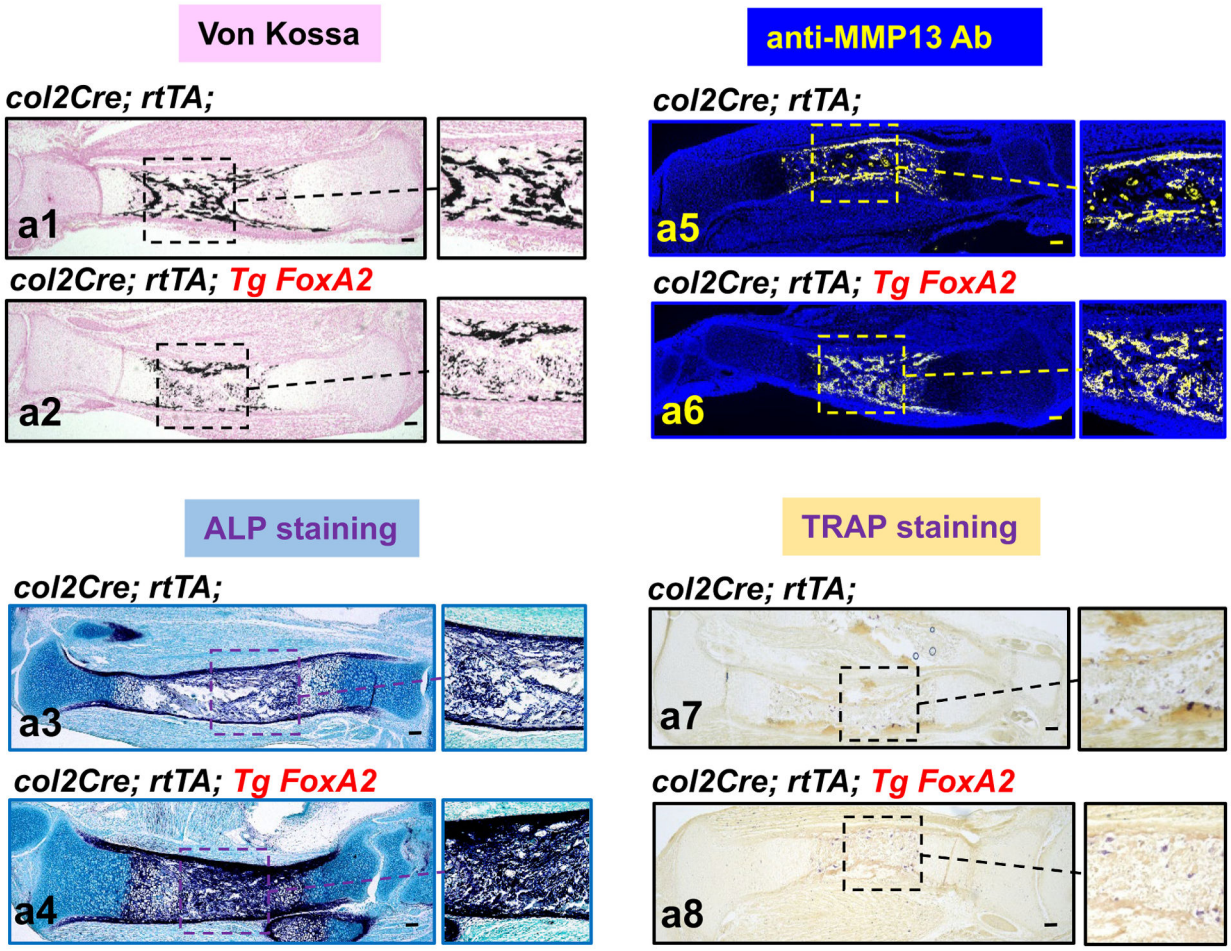

Fig. 4.

Less mineralization in the bone matrix of the transgenic mice. Von Kossa staining (a1–2), alkaline phosphatase activity (a3–4), immunohistochemistry for MMP13 (a5–6), TRAP staining (a7–8), in hindlimbs from control *col2CRE;Rosa26*$^{rtTA/+}$ mice (a1, a3, a5, a7) and transgenic *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ mice (a2, a4, a6, a8). Scale bars, 100 μm.

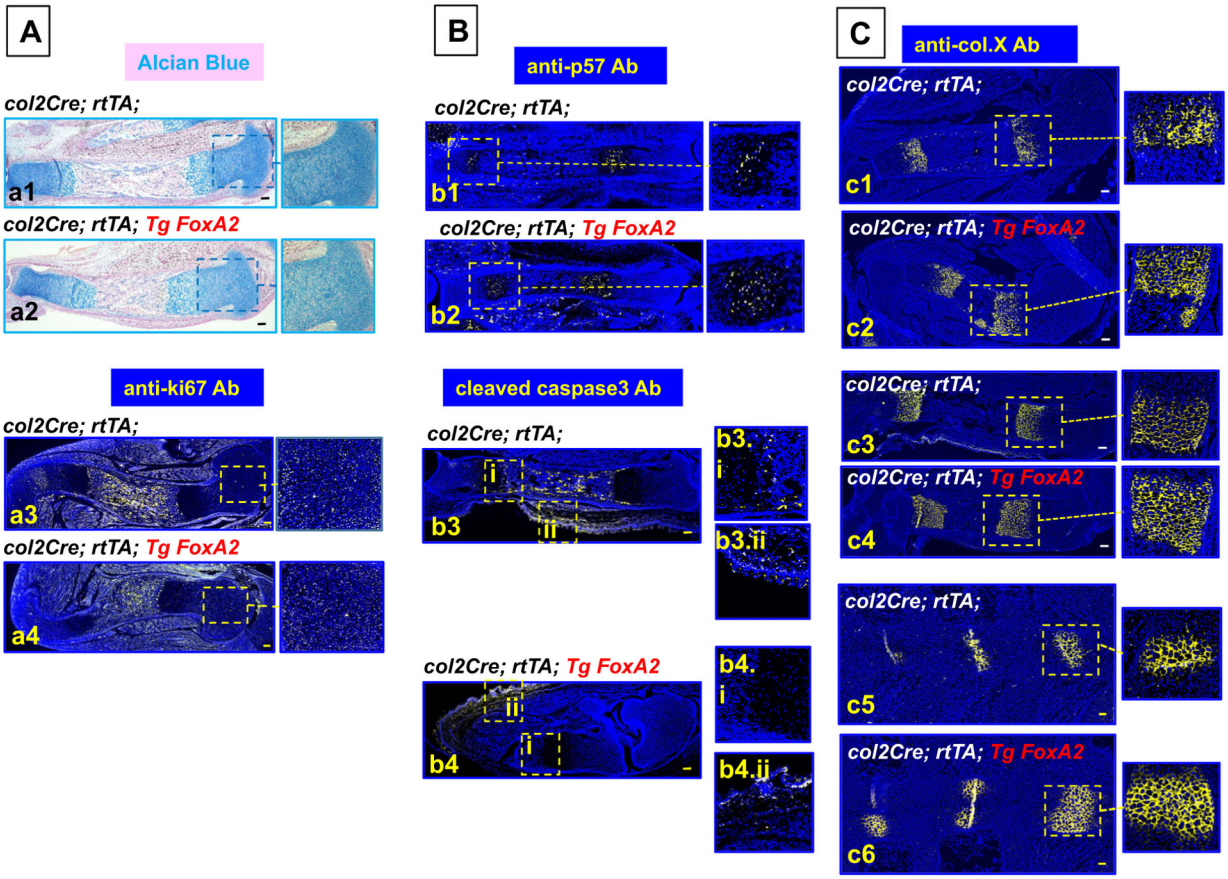

Fig. 5.

Overexpression of FoxA2 causes an accumulation of collagen 10+ hypertrophic chondrocytes, but it cannot trigger ectopic hypertrophy in the immature chondrocytes. Alcian Blue staining (a1–2), immunohistochemistry for Ki67 (a3–4) in hindlimbs from control *col2CRE;Rosa26*$^{rtTA/+}$ mice (a1, a3) and transgenic *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ mice (a2, a4) (A). Immunohistochemistry for p57 (b1–2) and cleaved caspase 3 (b3–4), in hindlimbs from control *col2CRE; Rosa26*$^{rtTA/+}$ mice (b1, b3) and transgenic *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ mice (b2, b4) Representative details, from each zone, are shown in numbered insets (i = hypertrophic zone/metaphyseal bone, ii = epithelial tissue) (B). Immunohistochemistry for collagen 10 (c1–6) in forelimbs (c1–2), hindlimbs (c3–4) and sterna (c5–6) from control *col2CRE;Rosa26*$^{rtTA/+}$ mice (c1, c3, c5) and transgenic *col2CRE;Rosa26*$^{rtTA/+}$*;TgFoxA2*$^{+/-}$ mice (c2, c4, c6) (C). Scale bars, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

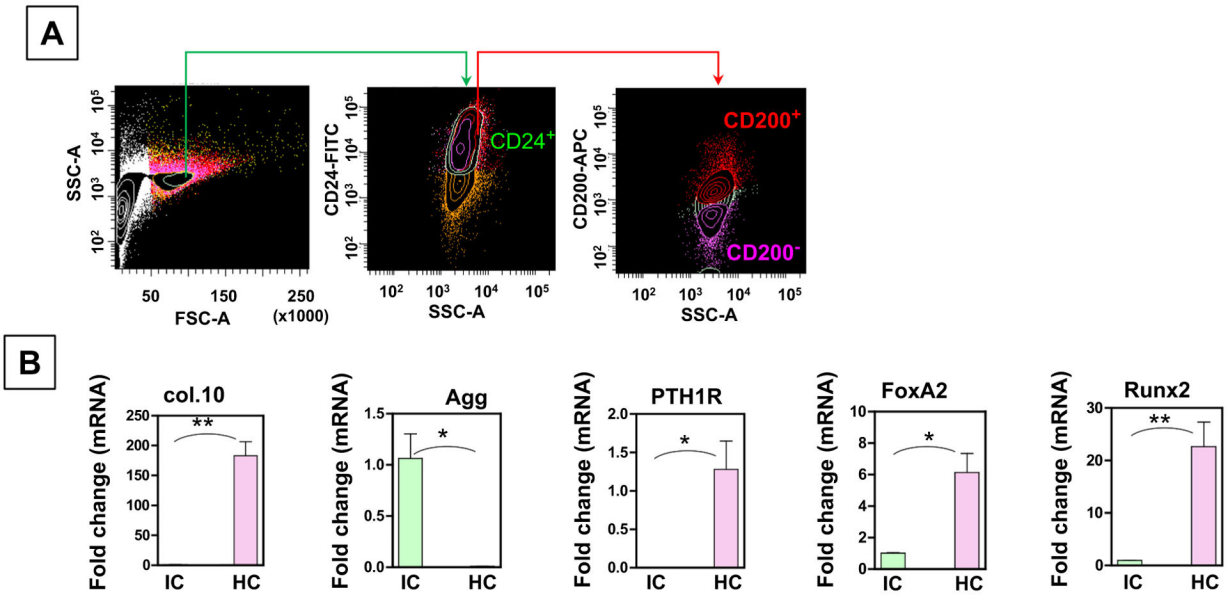

Fig. 6.

Separation of immature chondrocytes from hypertrophic chondrocytes. Separation by fluorescence-activated cell sorting (FACS) of two chondrocyte sub-populations: $CD24^{+}$ $CD200^{+}$ hypertrophic chondrocytes and $CD24^{+}CD200^{-}$ immature chondrocytes (A). RT-PCR for evaluating gene expression for *collagen 10*, *Aggrecan*, *PTH/PTHrP receptor*, *FoxA2*, and *Runx2* in $CD24^{+}$ $CD200^{+}$ hypertrophic chondrocytes (HC) and $CD24^{+}CD200^{-}$ immature chondrocytes (IC). Statistical significance is indicated with * denoting $p < 0.05$ or ** denoting $p < 0.01$. Significance assessed by student's *t*-test. (B).

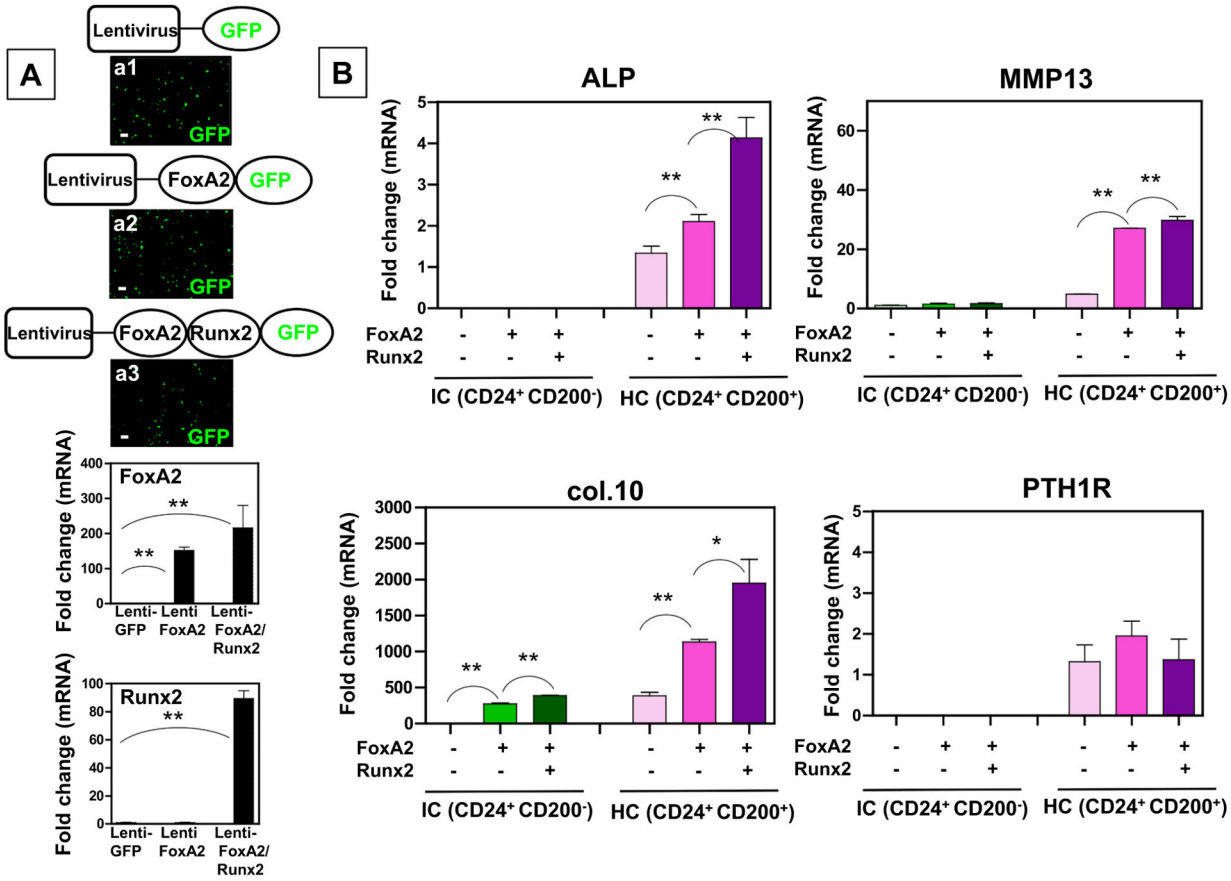

Fig. 7.

Lentiviral FoxA2 infection enhanced the expression of hypertrophic markers in differentiated chondrocytes, but it did not trigger ectopic hypertrophy in the immature cells. Infection of growth plate chondrocytes with a virus encoding either control GFP (Lenti-GFP), FoxA2 alone (Lenti-FoxA2-GFP) or FoxA2 in combination with Runx2 (Lenti-FoxA2-Runx2-GFP) and fluorescence microscopy for GFP expression (a1–3). Scale bars, 100 μm. RT-PCR for evaluating gene expression for *FoxA2* and *Runx2* in chondrocytes (A). RT-PCR for evaluating gene expression for *alkaline phosphatase (ALP)*, *MMP13*, *collagen 10*, and *PTH/PTHrP receptor* in $CD24^+$ $CD200^+$ hypertrophic chondrocytes (HC) and $CD24^+CD200^-$ immature chondrocytes (IC) (B). Statistical significance by t-test is indicated with * denoting $p < 0.05$ or ** denoting $p < 0.01$.