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The transcriptional cofactor Lbh regulates angiogenesis and endochondral bone formation during fetal bone development

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Abstract

Lbh is thought to act as a transcriptional cofactor and is highly conserved among species. Here we show that Lbh is expressed in chondrocytes, cells of the perichondrium, and the primary spongiosa in fetal growth plates of mice and chickens. Lbh overexpression in chick wings, using the RCAS-retroviral vector strategy, results in shortened skeletal elements and delayed hypertrophic chondrocyte maturation and bone formation. Additionally, osteoclast and endothelial cell invasion are delayed in the Lbh-overexpressing bones. Finally, we find a dramatic suppression of Runx2 and VEGF mRNAs in chondrocytes and osteoblasts that overexpress Lbh. Strikingly, this abnormal bone development in infected limbs can be rescued by concurrent overexpression of Runx2. These results suggest that during endochondral bone formation, Lbh may negatively regulate vascular invasion and formation of the early ossification center at least in part by interfering with Runx2 and/or VEGF expression.

Keywords

endochondral bone formation; Runx2; Lbh; VEGF

Introduction

In endochondral bone development mesenchymal condensates differentiate into chondrocytes that proliferate to form and enlarge the bone structure (Erlebacher et al., 1995; Karsenty, 2001; Kronenberg, 2003). Over time, cells in the center stop proliferating, become hypertrophic (named hypertrophic chondrocytes) and generate the surrounding matrix. They express predominantly type X collagen (col X), attract blood vessels and induce adjacent perichondrial cells to become osteoblasts, leading to the formation of a bone collar. With vascular invasion, osteoblasts appear in the primary spongiosa (PS) and there begin to synthesize new bone. Chondrocytes continue to proliferate and undergo well-ordered phases of maturation. This region of chondrocyte proliferation and maturation adjacent to the PS is often called the fetal growth plate. In long bones of the limbs, growth plates are detectable around embryonic day

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(E)14.5 in mice and around Hamburger-Hamilton (HH) stage 36 (E10) in chickens. The chondrocytes undergo cycles of proliferation, flatten and form orderly columns, become hypertrophic and eventually die, to be replaced by osteoblasts adjacent to the growth plate in the marrow space and the bone collar. While undergoing the different maturation stages, chondrocytes respond to a variety of signals to activate specific transcription factors (TF) that direct this maturation.

One crucial and well-characterized TF in the process of differentiation of both chondrocytes and osteoblasts is Runx2, which is mainly expressed in pre-hypertrophic and hypertrophic chondrocytes, as well as in perichondrial cells and osteoblasts (Karsenty, 2001; Komori, 2003). Loss of Runx2 in genetically engineered mice severely delays chondrocyte maturation and suppresses osteoblast development (Inada et al., 1999; Kim et al., 1999; Komori et al., 1997; Otto et al., 1997). Conversely, transgenic expression of Runx2 specifically in chondrocytes accelerates chondrocyte hypertrophy (Takeda et al., 2001; Ueta et al., 2001). Additionally, Runx2 is a necessary component of a tissue-specific genetic program that regulates production of vascular endothelial growth factor (VEGFA, here called VEGF) (Zelzer et al., 2004). Runx2-deficient mice present an almost complete lack of VEGF expression in hypertrophic chondrocytes as well as a dramatic decrease in levels of the VEGF receptors in perichondrial cells (Zelzer et al., 2001). Partial loss of VEGF proteins in mice impairs skeletal angiogenesis, delays chondrocyte hypertrophy, bone formation and cartilage calcification (Maes et al., 2002; Zelzer et al., 2002).

To identify novel regulators of chondrocyte differentiation, we analyzed the genes expressed in discrete layers (round, flat and hypertrophic cell layers) of newborn (NB) wild-type (WT) mice growth plates by microarray analysis (Nishimori et al, manuscript in preparation), focusing on genes differentially expressed across the growth plate. We found that the transcriptional cofactor limb-bud-and-heart (Lbh) is weakly expressed in round and flat chondrocytes, but strongly expressed in late hypertrophic chondrocytes and osteoblasts. Lbh was discovered as a small acidic nuclear protein highly conserved among species (Xenopus, Mammals, Avians, Humans) (Briegel et al., 2005; Briegel and Joyner, 2001). The name Lbh was given to the protein because of its prominent expression in fetal life in the early limb bud and heart. Lbh has no known DNA binding domain, though it can activate expression of a reporter gene when fused to the DNA-binding domain of GAL4 (Briegel and Joyner, 2001). In humans the Lbh gene is located on chromosome 2p. In mice, the transgenic expression of Lbh in the heart from the three somite stage onwards leads to a broad spectrum of cardiovascular defects mimicking the partial trisomy 2p syndrome described in humans (Briegel et al., 2005). In endochondral bone formation, the role of Lbh is completely unknown. In case reports of human fetuses with partial trisomy 2p syndrome, skeletal phenotypes such as polydactyly and scoliosis have been described (Hahm et al., 1999). Since Lbh in fetal/newborn bones in mice is mainly expressed in late hypertrophic chondrocytes and osteoblasts, we hypothesized that Lbh might play a role in hypertrophic chondrocyte maturation and/or formation of the primary ossification center (POC).

Here, we demonstrate the expression of Lbh transcripts in developing chondrocytes, cells of the perichondrium and of the POC in both mice and chicken fetal growth plates. Forced expression of Lbh in developing chicken wings impairs chondrocyte hypertrophy, growth plate vascularization, bone formation and osteoclast invasion. Furthermore, forced expression of Lbh leads to a dramatic decrease of Runx2 and VEGF mRNA expression in multiple cell types in developing limbs. Comparable effects were observed after forced Lbh expression in chick primary chondrocytes and in murine chondrogenic and osteogenic cell lines. Double-overexpression of Lbh and Runx2 in avian *in vivo* and *in vitro* models rescues the Lbh-induced phenotype. These findings suggest a possible role of Lbh in the tight regulation of Runx2 transcription as well as a possible role of Lbh in the regulation of VEGF mRNA expression

during endochondral bone development. Thus Lbh has previously unrecognized crucial roles in chondrocyte hypertrophy, vascular invasion and osteoblast maturation.

Materials and Methods

Generation of RCAS-Lbh retroviruses and retroviral overexpression

RCASBP(A)-Lbh and RCASBP(B)-Lbh were generated by RT-PCR (for cloning details see supplementary data). Infective viruses were generated as described elsewhere (Logan and Tabin, 1998). RCAS viruses were micro-injected into chicken limb buds at embryonic stage HH18-20 (E3–E3.5) *in ovo* and the embryos were further incubated until the designated stage between HH33 and HH42 (E7.5–E13). In each experiment the infected wing was compared to the uninfected wing of the same animal that served as the "WT"- control. For our rescue experiments we took advantage of the possibility to co-overexpress two different genes in the same cells using two RCAS viruses of different subgroups A and B.

In situ hybridization (ISH)

Details of the probes employed for ISH are available upon request. Paraffin sections of embryonic chick and mouse tissue were prepared as described (Murtaugh et al., 1999). Non-radioactive ISH, with digoxigenin (DIG)-labelled probes (Murtaugh et al., 2001) as well as ISH with ^{35}S-labeled riboprobes was performed as described previously (Chung et al., 1998). Autoradiography, Hoechst 33258 staining (Invitrogen) as well as photography has been performed as described elsewhere (Sundin et al., 1990).

Tissue culture experiments

Chicken upper sternal chondrocytes were harvested and cultured as described (Grimsrud et al., 1999; Usui et al., 2008). The primarily isolated cells were plated in 100 mm tissue culture dishes at a density of 3×10^6 cells and cultured for 5 days when the chondrocytes were in the supernatant. Then the chondrocytes were harvested and retroviral infection performed with an empty RCAS virus (RCAS-EV), RCAS-GFP, RCAS-Lbh and RCAS-Runx2 before starting the secondary cultures in 12-well plates at a density of 11×10^5 cells. The cells were cultured for 48 hours to 7 days for gene expression experiments.

Mouse chondroblastic cells of the ATDC5 cell line and mouse osteoblastic cells of the MC3T3 cell line were used. ATDC5 cells were cultured in a 1:1 mixture of DMEM and Ham's F-12 medium (DMEM/F-12; Invitrogen) containing 5% FBS (Invitrogen), 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen). For induction of differentiation, media were supplemented with 10 μg/ml human transferrin, 3×10^{-8} M sodium selenite and 10 μg/ml bovine insulin (ITS) (Roche). MC3T3 cells were cultured in αMEM (Invitrogen) containing 5% FBS (Invitrogen), 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen). For induction of mineralization, media were supplemented with β-glycerol phosphate (10 mM) and ascorbic acid (50 μg/ml) (Sigma).

For stable transfections, ATDC5 cells and MC3T3 cells were plated at a density of 2×10^5 cells/cm^2. After 24 h, they were stably transfected with pcDNA3.1 containing the full-length coding region of Lbh (generously provided to us by Dr. Karoline Briegel, University of Miami) (Lbh) (Briegel et al., 2005) or with the empty vector (EV, Invitrogen) using the Effectene transfection reagent (QIAGEN) according to the manufacturer's instructions. After 24 h, and every 48 h thereafter for 2 weeks, media were replaced with fresh media containing 300 μg/ml of G418. This dose and duration of treatment resulted in the death of all nontransfected cells within 10 days. Pools of 12 clones of Lbh and EV were isolated for further studies.

Quantitative Reverse Transcriptase-PCR

Total RNA was extracted from isolated and crushed carpometacarpals and ulnae of HH39 old control and RCAS-Lbh overexpressing chicken wings by using the RNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. cDNA synthesis was performed by using random hexamers with the Protoscript First Strand cDNA Synthesis Kit (New England Biolabs), and quantitative PCR was performed by using DNA Engine Opticon 2 Continuous Fluorescence Detection System (Biolab, Boston, MA) and the Sybr green mix (Applied Biosystems, Lincoln, CA). Signals were normalized to either chicken GAPDH (Dong et al., 2006) or mouse β-actin (Bastepe et al., 2004). Each reaction was performed in quadruplicate and repeated on different sample sets to confirm the results. For primer details, see supplementary data.

Statistical analyses

Date are expressed as mean ± SEM. Statistical significance was assessed by Student`s T test. Values were considered statistically significant when $p<0.05$.

Results

Lbh expression in skeletal development suggests a role in cartilage and bone formation

While analyzing the expression of TF in the discrete layers of the mouse newborn growth plate using microarray analysis and quantitative real-time reverse transciptase polymerase chain reaction (QRT-PCR), we found that Lbh was expressed in round, flat but predominantly hypertrophic chondrocytes (data not shown). To further characterize Lbh expression we performed in-situ hybridization (ISH) analysis. In E14.5 mouse embryos Lbh mRNA was highly expressed in round chondrocytes (Figure 1A). In newborn (NB) mice we found Lbh mRNA expressed in round, flat and late hypertrophic chondrocytes, as well as cells of the perichondrium and the POC (Figure 1B, Figure S4). We also analyzed Lbh expression in chicken wings at comparable stages of development. Altogether, the Lbh mRNA expression resembled the expression pattern seen in mouse development. In chicken wings at HH39 (roughly equivalent to E11 in the limb growth plate), a developmental time when the fetal growth plate is formed and the POC appears, we found Lbh mRNA expressed in round, flat, and late hypertrophic chondrocytes, as well as in the perichondrium and POC (Figure 1C). In the chick the late hypertrophic layer is more prominent than in the mouse, making the expression in this layer particularly clear. High levels of Lbh transcripts were also found in surrounding limb tissue in both mice and chickens.

Lbh overexpression during chicken wing development impairs hypertrophic chondrocyte maturation, vascular invasion, and bone formation

The distribution of Lbh mRNA expression in the fetal growth plate of the developing endochondral bone suggests a potential role for Lbh in the control of chondrocyte proliferation and maturation, and bone formation. To further investigate this hypothesis we infected chicken wing buds with the Replication-Competent ASLV long terminal repeat with Splice acceptor (RCAS) retroviral vector type (A) encoding chicken Lbh to demonstrate its actions during cartilage and bone formation and growth. The RCAS vector type (B) encoding chicken Lbh was also used. Both Lbh-encoding vectors showed comparable results. Infections were performed at HH18-20 (E3-3.5) and the embryos were allowed to develop through HH33 and HH42 (E7.5–E13), stages at which hypertrophic chondrocytes and the bone collar are normally seen. Skeletal preparation of RCAS-Lbh overexpressing wings of E11 (HH39) chicken embryos revealed that the long bones were normally shaped; however, both the overall lengths of the bones and the zones of mineralization (marked by alizarin red staining) were shorter when infected wings were compared to the control wings (Figure 2A). The difference in the

lengths of humerus, ulna and carpometacarpal between control and infected limbs is particularly statistically significant. This suggests a delay in endochondral ossification (Figure 2A).

Histological analysis demonstrated that in HH33 (E7.5) uninfected contralateral control carpometacarpal bone, hypertrophic chondrocytes are present in the center of the developing cartilage but not in RCAS-Lbh overexpressing bones (Figure 2Ba+b, Figure 2Bc+d).

In chicken carpometacarpals vascular invasion normally starts around HH37 (E10.5) (Figure 2Bc), followed by vascular ingrowth, cartilage resorption and the beginning of the formation of the POC (Figure 2Bg). Again, this whole process was delayed in the RCAS-Lbh overexpressing wings at comparable stages (Figure 2Bd, f, h). This phenotype was observed in 98% of RCAS-Lbh-infected wings (n>90) with mild variations in the phenotypic expression, perhaps due to variation of the infection efficiency or variable age at the time of infection. Control experiments using an RCAS-GFP virus revealed a comparable and normal development of both control and infected wings, thereby excluding a viral nonspecific and/or manipulation-induced effect (Figure S1A). The overexpression efficiency was tested by ISH analyzes for Lbh mRNA expression (Figure S1B) as well as QRT-PCR analysis (Figure 6A). Over time, development of the RCAS-Lbh overexpressing wings caught up with that of control wings after a delay of 1–2 days (Figure 2C).

The delayed hypertrophic chondrocyte maturation, vascular invasion and bone formation led us to assess whether the regulation of chondrocyte and/or osteoblast development were disturbed. Therefore, we investigated the expression of stage-specific differentiation markers of these cell types using ISH. Strikingly, at stage HH33, at which hypertrophic chondrocytes are visible in the carpometacarpals of control but not RCAS-Lbh overexpressing wings, there was also no detectable expression of the hypertrophic chondrocyte marker collagen X mRNA (col X) in RCAS-Lbh infected wings. In contrast, both control and RCAS-Lbh overexpressing wings revealed normal expression of the proliferating chondrocyte marker, collagen II mRNA (col II) (Figure 3Aa–d). ISH analyses of later stages also showed a normal distribution of transcripts for proliferating chondrocytes marked by col II and for prehypertrophic chondrocytes marked by Indian hedgehog (Ihh) (Figure 3Ae–h). Somewhat surprisingly, given the normal distribution of proliferative chondrocyte markers, BrdU labeling of RCAS-Lbh infected wings revealed a higher proliferation rate of periarticular cells and a higher cell number in RCAS-Lbh overexpressing chicken wings (Figure S2). Comparisons of proliferation rates at varying time points shows that this increase in proliferation is not caused by a delay in the state of the proliferating chondrocytes in the RCAS-Lbh-infected wings. Consistent with the eventual appearance of hypertrophic chondrocytes in the RCAS-Lbh overexpressing wings over time, by stage HH36, col X is expressed in a pattern similar to that of the control wing (Figure 3Ai+j). Interestingly, however, expression of the late hypertrophic marker osteopontin (OP) is dramatically reduced and restricted to a smaller domain in the RCAS-Lbh overexpressing bone than in the control wing. These data demonstrate that overexpression of Lbh in chicken wings delays both early and late hypertrophic chondrocyte maturation.

We next looked at osteoblast maturation and studied markers of early and mature osteoblasts via ISH analyses. Expression of collagen I mRNA (col I), a marker of both early and mature osteoblasts, was found in both control and RCAS-Lbh overexpressing bones, but the domain of expression was less extensive and the intensity of expression were much less in the infected bones (Figure 3Ak+l). Lbh overexpression also downregulated OP mRNA expression in the bone collar a molecule produced by more mature osteoblasts (Figure 3Am+n). These findings were confirmed by QRT-PCR analysis of expression of various genes associated with late hypertrophic chondrocyte and osteoblast development, using isolated and crushed carpometacarpals and ulnae of HH39 old control and RCAS-Lbh overexpressing chicken

wings: OP, osteocalcin (OC) and MMP13 (synthesized by both late hypertrophic chondrocytes and osteoblasts) mRNA levels were significantly reduced in RCAS-Lbh overexpressing bones. Furthermore, the level of col I mRNA was reduced, though the difference was not statistically significant. Taken together, these data suggest that overexpressing Lbh in chicken wings leads to a delay in hypertrophic chondrocyte development, as well as a delay in osteoblast development.

Bone collar formation, cartilage resorption, as well as osteoclast and endothelial cell invasion are delayed in RCAS-Lbh overexpressing chicken wings

To further investigate the phenotype observed in Lbh-overexpressing wings, we analyzed the bone mineralization of control and RCAS-Lbh overexpressing carpometacarpals and ulnae at different time points. In normal endochondral bone development, mineralization of bone starts in the perichondrial region. This occurs around stage HH34 (E7) in chick ulnae. At this stage, we observed a mineralized bone collar surrounding the midshaft cartilage (Figure 4Aa) in the control bone. Strikingly, this primarily mineralization process could not be detected in the RCAS-Lbh overexpressing ulna (Figure 4Ab). Over time, we observed delayed bone collar formation in the RCAS-Lbh overexpressing wing. At stage HH39 of control and RCAS-Lbh overexpressing carpometacarpals, we still saw an underdeveloped bone collar in the infected bone, as reflected by its decreased length and thickness (Figure 4Ac+d). We conclude that RCAS-Lbh overexpression resulted in a delay of bone collar formation preceding the initial capillary invasion.

Vascular invasion is associated with cartilage resorption in the core of the endochondral developing bone. At the border between the growth plate and the POC, chondrocytes undergo apoptosis. Given the delay in mineralization and vascular invasion in RCAS-Lbh overexpressing bones, we investigated whether cellular apoptosis might also be affected. In control bones apoptotic cells detected by TUNEL assay were found in the terminal row of chondrocytes adjacent to the invading blood vessel as well as in the POC. At comparable stages in the RCAS-Lbh overexpressing bones, apoptotic cells appear later (Figure 4 B), consistent with the delay of vascular invasion in RCAS-Lbh overexpressing bones.

Next we analyzed the cellular correlates of the impaired cartilage resorption by assessing markers of osteoclast and endothelial cell invasion. At the stage of vascular invasion in the control bones, multinucleated TRAP-positive osteoclasts as well as CD31 positive endothelial cells appeared in the centers of the bones' midshafts, where blood vessel invasion occurred (Figure 4C, Figure 4D). In contrast, in RCAS-Lbh overexpressing bones, multinucleated TRAP-positive osteoclasts were located along the diaphysis, where they appeared to be lined up at the surface of the bone but were not yet penetrating into the midshaft center (Figure 4C). Furthermore, there were fewer CD31 positive endothelial cells, and they were found similarly lined up along the diaphysis (Figure 4D). Consistent with the delay in the resorption process, levels of mRNA encoding MMP9, a key regulator of growth plate angiogenesis and hypertrophic chondrocyte apoptosis expressed in osteoclasts and endothelial cells (Vu et al., 1998), were 2-fold reduced in RCAS-Lbh overexpressing bones at HH39 (E11) (Figure 5E). Taken together, these findings indicate that RCAS-Lbh overexpressing chicken wings exhibited a delay in osteoclast and endothelial cell invasion.

Lbh represses Runx2 and VEGF mRNA expression

We wondered whether Lbh overexpression inhibits chondrocyte hypertrophy by interfering with the expression of Runx2, a necessary regulator of chondrocyte hypertrophy. Further, Runx2 also regulates the expression of vascular endothelial growth factor (VEGF) in hypertrophic chondrocytes (Zelzer et al., 2004); this VEGF has been shown to play a crucial role in regulating the formation of the POC (Carlevaro et al., 2000; Deckers et al., 2000; Engsig

et al., 2000; Gerber et al., 1999). Decreased VEGF expression leads to a phenotype that resembles the phenotype seen in our overexpression studies (Gerber et al., 1999; Maes et al., 2002; Zelzer et al., 2002).

We therefore examined the expression of Runx2 and VEGF mRNA via ISH analysis at stages HH34-39 (E9-11). We found that overexpressing Lbh dramatically suppresses levels of Runx2 and VEGF mRNAs in chondrocytes and osteoblasts in comparison to levels in the control bones (Figure 5A, Figure S5). The mRNAs were suppressed in both the bone and in the adjacent soft tissues; the extent of suppression could not be explained simply by stage-appropriate changes in Runx2 expression (compare expression of Runx2 in Figure 5A and Figure S5). This was confirmed in QRT-PCR analysis of control and Lbh-overexpressing bones at stage HH39 (Figure 5B). These data suggest that Runx2 and/or VEGF downregulation might be responsible for the Lbh-induced phenotype. To test this hypothesis, we asked whether overexpression of Runx2 could rescue the Lbh-induced delay in hypertrophic chondrocyte maturation, vascular invasion and bone formation. We co-infected HH18-20 limb bud with either the viruses RCAS (B)-Lbh and RCAS(A)-Runx2 (n=12), or the viruses RCAS(A)-Lbh and RCAS(B)-Runx2 (n=5). Chicken embryos were allowed to develop until HH39 (E11). Together, both injection strategies resulted in reversal of the abnormal phenotype in 92% (15 vs. 17) of the bones studied. We injected viruses at stage HH18-20 (E3-3.5) and the embryos were allowed to develop through HH39 (E11). As expected, in control experiments (Figure 5C), we observed delay in late hypertrophic chondrocyte maturation, vascular invasion and formation of the POC when RCAS(A)-Lbh virus was co-infected along with a control RCAS(B)-GFP virus. The infection efficiency of both Runx2 and Lbh was tested by ISH (Figure S3A). Single RCAS-Runx2 overexpression studies (n=6) showed moderately but consistently advanced vascular invasion, mineralization and formation of the POC (Figure S3B). Wings co-infected with RCAS-Lbh and RCAS-GFP showed the phenotype seen in single RCAS-Lbh overexpressing bones, with a shortened skeletal element and shortened zone of mineralization, a delayed formation of the POC and a repressed expression pattern of OP and VEGF mRNA (Figure 5Cb, e, h, k, n) at stage HH39 as compared with contralateral uninfected wings (control) (Figure 5Ca, d, g, j, m). Strikingly, when Runx2 was overexpressed along with Lbh, we observed a macroscopically and histologically normal looking bone with normal expression of OP and VEGF (Figure 5D c, f, i, l, o) in both late hypertrophs and osteoblasts, demonstrating that Runx2 overexpression rescues the Lbh-induced phenotype. Thus, retroviral-encoded Lbh inhibits chondrogenesis and osteogenesis by either directly or indirectly repressing the expression of both Runx2 and VEGF.

Misexpression of Lbh as well as misexpression of both, Lbh and Runx2 in avian and murine cell lines in vitro can reproduce parts of the phenotype seen in vivo

The studies so far involve infection of all cell types in the limb. To determine whether Lbh overexpression only in chondrocytes mimics the phenotypes seen in the chondrocytes of Lbh-overexpressing limbs, we overexpressed Lbh in isolated upper sternal chondrocytes, chondrocytes that differentiate into hypertrophic chondrocytes in culture and express hypertrophic chondrocyte specific markers like col X. Primary chondrocytes from the upper region from E16 WT chickens were isolated, infected *in vitro* with RCAS-Lbh, or RCAS-empty vector (EV) and cultured for an additional 7–14 d before harvesting. QRT-PCR analyses demonstrated the following results: Lbh was expressed at 6-fold higher levels in the RCAS-Lbh overexpressing cells as compared to the RCAS-EV infected control cells. Strikingly, there was a dramatic and significant downregulation of both Runx-2 and VEGF in the Lbh-overexpressing cells. Additionally, col X and OP expression were downregulated in the Lbh-overexpressing cells as compared to the EV-infected cells. We next assessed the effects of overexpressing both Lbh and Runx2 in primary chondrocytes. QRT-PCR analyses demonstrated that Runx2 overexpression rescued the decreased Runx2 and VEGF mRNA

levels seen in the single Lbh-overexpressing cells (Figure 6B). We conclude that Lbh overexpression directly in chondrocytes *in vitro* mimics the effects of Lbh overexpression in limbs *in vivo* on hypertrophic chondrocyte maturation. It is most likely that Lbh regulates this process via the regulation of Runx2 and VEGF mRNA expression, as a double-infection with Lbh and Runx2 could rescue the Lbh-induced decreased differentiation and downregulation of VEGF mRNA expression.

Since Lbh is found in mouse fetal growth plates in a distribution similar to that in chickens, we wondered if Lbh overexpression would have similar effects in a mouse chondrocyte model. To assess whether stable expression of Lbh could delay chondrocyte differentiation murine ATDC5 cells, a chondrocyte line that differentiates into hypertrophic chondrocytes *in vitro*, were stably transfected with pcDNA3.1-Lbh (Lbh) or pcDNA3.1-empty vector (EV). After 14 d in culture, the stably Lbh-transfected ATDC5 cells failed to show any alkaline phosphatase (AP) positive staining (a marker of hypertrophic chondrocytes), while the stably EV transfected-ATDC5 cells showed positive, blue AP staining of the cells (Figure 6Ca–d). Of note, cell confluence did not differ between both cell types (Figure 6Cd`). Next we examined the effect of stable expression of Lbh on mineralized matrix formation, a final step in chondrocyte differentiation. Alizarin Red positive nodules were detected after 30 days in culture only in the EV-transfected ATDC5 cell, while mineralized nodules were not detectable in the Lbh-transfected cells at that time (Figure 6Ce+f). At 7 d ATDC5-Lbh clones expressed 20.0 ± 0.2-fold higher Lbh mRNA levels than ATDC5-EV clones. As in avian cells, we observed a significant, approximately 80% downregulation of VEGF mRNA levels in the Lbh-transfected cells, as well as a approximately 70% downregulation of Runx2 mRNA levels compared to the EV transfected cells. Aggrecan mRNA, as a specific early chondrocyte marker was expressed without a difference in both Lbh- and EV-transfected ATDC5 cells (Figure 6E).

To determine whether the effects of Lbh overexpression in osteoblasts involves direct actions on osteoblasts, murine MC3T3 cells were used. These cells have characteristics of preosteoblasts with the capability to become mature osteoblasts in prolonged cultures at higher cell densities. MC3T3 cells were stably transfected with pcDNA3.1-Lbh (Lbh) or pcDNA3.1-empty vector (EV). After 14 d in culture, the EV-transfected MC3T3 cells started to express AP while this expression was still missing in the Lbh-transfected cells (Figure 6Da–d). To assess a possible impairment of differentiation as reflected in the ability to form mineralized bone nodules, we cultured MC3T3-Lbh and MC3T3-EV cells in mineralization medium for various times. At 14 days, the MC3T3-EV cells started to form mineralized nodules, while those could not be detected at the same time in MC3T3-Lbh cells (Figure 6De+f). The number of nodules was still strikingly decreased at 30 days in Lbh-transfected cultures compared to EV-transfected cultures (Figure 6Dg+h). To determine whether Runx2 and VEGF expressions were altered by forced expression of Lbh in MC3T3 cells, cells cultured for 7 days were harvested and examined by QRT-PCR analyzes. Again, both Runx2 and VEGF mRNA levels appeared to be dramatically and significantly downregulated in MC3T3-Lbh cells compared to MC3T3-EV cells, while col I mRNA was expressed at a similar level in both EV and Lbh transfected cells (Figure 6F). The findings demonstrate that in both avian and murine cells, the forced expression of Lbh leads to a delay in chondrocyte and osteoblast development with a delay of mineralization. The *in vitro* studies suggest that the effects of Lbh overexpression in chondrocytes and osteoblasts are cell-autonomous actions of Lbh in each cell type.

Discussion

Recent work by a number of groups begun to clarify the process of late hypertrophic chondrocyte maturation, vascular invasion and bone formation. These processes involve mechanisms linking Runx2 and VEGF regulation (Papachristou et al., 2005; Zelzer et al., 2001). In this report, we have uncovered a possibly crucial novel regulator of this process: Lbh.

This study indicates that expression of Lbh can influence normal angiogenesis, endochondral ossification, and bone formation at least partly through regulation of Runx2 mRNA expression.

Lbh overexpression in developing chondrocytes and osteoblasts leads to three defects that we demonstrate: first, a delay in initial hypertrophy; second, delays in late hypertrophy and vascular invasion; and third, defects in mineralization of bone. The consequences of Lbh overexpression might result exclusively from actions on early hypertrophic chondrocytes, as these cells control all the subsequent steps in endochondral bone development. Several arguments suggest, however, a series of independent actions of Lbh. First, the delay in late hypertrophy is associated with dramatic and prolonged suppression of OP and VEGF mRNA that appear out of proportion to the hypothesis of simple delay in the generation of late hypertrophic chondrocytes. Similarly the bone defect is more pervasive and is accompanied by widespread defects in Runx2 expression in the soft tissues in a way that cannot easily be explained by poor signalling from hypertrophic chondrocytes. Most importantly for this argument, the actions of Lbh on isolated MC3T3 osteoblastic cells sufficiently resemble those in the osteoblasts of the infected chick wings that direct actions of Lbh on both chondrocytes and osteoblasts seem likely.

Suppression of Runx2 mRNA most likely explains the defects seen in infected hypertrophic chondrocytes. In chickens and mice, Runx2 as well as Runx3 are required for chondrocyte hypertrophy (Komori, 2003) and overexpression of Runx2 leads to exaggerated hypertrophy (Enomoto et al., 2000). The rescue of the Lbh-overexpression phenotype through overexpression of Runx2, thus, strongly supports the idea that suppression of Runx2 expression explains the hypertrophic chondrocyte phenotype caused by Lbh overexpression. Further studies will be required to determine whether the Runx2 gene is a direct target of Lbh or instead whether Lbh acts upstream of the decision to activate Runx2 in chondrocytes.

The defect in osteoblast development, vascular invasion and mineralization is most likely a consequence of the downregulation of Runx2 and VEGF mRNA expression in both the chondrocytes and osteoblasts in the Lbh-overexpressing limbs. Runx2 is required for osteoblast development in mice (Karsenty, 2001) and also regulates the expression of VEGF (Zelzer et al., 2001). Thus, the decrease in VEGF expression seen with Lbh overexpression could reflect actions of Lbh on Runx2 expression and consequent decrease in VEGF expression or independent actions on the expression of both Runx2 and VEGF.

VEGF is a major regulator of vascular invasion in developing cartilage (Zelzer et al., 2004) and both VEGF and Runx2 regulate osteoblast development through actions on osteoblasts (Maes et al., 2002; Zelzer et al., 2004; Zelzer et al., 2002). Studies of VEGF loss of function demonstrate an abnormal process of hypertrophic chondrocyte maturation, vascular invasion and bone formation (Gerber et al., 1999; Maes et al., 2002; Zelzer et al., 2002). Those animals, like our Lbh-overexpressing chicken wings, presented shorter and less mineralized bones, as well as decreased expressions of col X, OP, OC, col I, CD31 and MMP9.

Nevertheless, in contrast to what we have reported here, animals with impaired VEGF protein expression or activity in the growth plate show an enlargement of the hypertrophic chondrocyte zone that we did not observe in the Lbh-overexpressing chicken wings. A possible explanation for this is that decreased Runx2 levels may balance the increased accumulation of chondrocytes expected from decreased VEGF expression. Without much Runx2 the slowness in making hypertrophic chondrocytes and the pervasive defects in late hypertrophy may mean that the generation of late hypertrophic chondrocytes is not fast enough to lead to major expansion of these cells in Lbh-overexpressing bones. It is also possible that the loss of VEGF protein can be mitigated by actions of circulating VEGF. Together, these data suggest that decreased VEGF levels combined with decreased Runx2 mRNA levels may explain most of the consequences

of Lbh overexpression in chick wings. Our studies have focused on roles of Lbh in hypertrophic chondrocytes and osteoblasts. However, Lbh is also expressed in proliferating chondrocytes. Perhaps this expression has a role in stopping expression of Runx2 in these early chondrocytes. Further, though the proliferative layer of chondrocytes appears relatively unperturbed in Lbh-expressing limbs, with normal morphology and expression of col II mRNA, these chondrocytes do exhibit a modest but definite increase in proliferation rate, as reflected by BrdU incorporation. Further studies will be needed to define better the roles of Lbh in proliferating chondrocytes.

However, given the dramatic reduction in VEGF expression in our overexpression studies, we would have expected a more dramatic phenotype with much shorter and misshaped skeletal elements in the Lbh-infected bones. Nevertheless, our phenotype caught up in 1 to 2 days and the bones never appeared malformed or misshaped. This is a very interesting and unexpected observation. Again, one explanation might be that circulating VEGF might rescue the local lack of VEGF protein and therefore normalize the developmental status in the Lbh-overexpressing bone. There are other possible explanations, as well. Runx3 expression mitigates the effects of Runx2 knockout in some growth plates of the mouse; perhaps Runx3 can also mitigate effects of Runx2 in the chick (but not the suppression of VEGF expression).

Further, even though the suppression of Runx2 mRNA is visually dramatic when examined by ISH, the quantitative RT-PCR makes clear that the suppression of Runx2 mRNA is not complete and would not be expected to duplicate the precise findings of the mouse knockout. Furtehr, we should acknowledge that the actions of the virus may change over time in ways that we did not fully monitor; this, too, could explain the paradox of vivid suppression of Runx2 mRNA at the times checked and the modest net phenotype over time. Finally, it is also possible that Lbh has other actions besides suppressing Runx2 and some of those actions may actually lead to a phenotype that is more normal than the loss of Runx2 would predict.

Lbh is expressed in both mouse and chicken fetal growth plates with a comparable expression profile: Transcripts appear in proliferative chondrocytes, cells of the perichondrium, late hypertrophic chondrocytes, and osteoblastic cells of the PS. Moreover, chicken primary chondrocytes as well as murine chondrocytes and osteoblasts express Lbh in comparable levels. Because of similar results in both murine and chicken *in vitro* cell models, as well as in chick limbs *in vivo*, we hypothesize that Lbh, at least in higher vertebrates, might have evolutionarily conserved functions. The conserved sites of Lbh expression match well with the sites of action that we observed in the overexpression studies. As Runx2 is downregulated in infected chondrocytes and osteoblasts, we speculate that, in the normal setting, Lbh suppresses expression of Runx2. In proliferative chondrocytes, Lbh might be a partner of other regulators that prevent Runx2 expression in these cells and allow them to proliferate normally. The decrease in Lbh expression in hypertrophic chondrocytes may be required for optimal expression and action of Runx2 in these cells. In late hypertrophic chondrocytes and osteoblasts, where Lbh and Runx2 are both expressed, Lbh might be a negative regulator of Runx2 expression, preventing undesirable consequences of Runx2 overexpession (Enomoto et al., 2000).

The putative role of Lbh as transcriptional cofactor has been discussed in an Lbh gain of function experiment using transgenic mice that overexpress Lbh in the heart (Briegel et al., 2005). In those mice, Lbh may act as a trans-acting modulator of key cardiac transcription factors, as Lbh overexpression in tissue culture cells predominantly represses Nkx2.5 and Tbx5, two key-modulators of cardiac development. It would be pleasing to propose a uniform mechanism whereby Lbh regulates different key transcription factors both in heart and bone, but the paucity of biochemical analysis makes that currently impossible. Further studies, along

with much needed gene ablation experiments will be required to establish the importance and mechanism of action of this important gene regulator.

In summary, we have shown that Lbh delays chondrocyte hypertrophy and negatively regulates late hypertrophic chondrocyte maturation, bone formation and vascular invasion in the fetal growth plate. These actions are associated with suppression of Runx2 and VEGF mRNA and can be reversed by forced expression of Runx2. These and previous studies (Briegel et al., 2005) suggest that Lbh is an important regulator of both cardiac and bone development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Afzal F, Pratap J, Ito K, Ito Y, Stein JL, van Wijnen AJ, Stein GS, Lian JB, Javed A. Smad function and intranuclear targeting share a Runx2 motif required for osteogenic lineage induction and BMP2 responsive transcription. J Cell Physiol 2005;204:63–72. [PubMed: 15573378]

Bastepe M, Weinstein LS, Ogata N, Kawaguchi H, Juppner H, Kronenberg HM, Chung UI. Stimulatory G protein directly regulates hypertrophic differentiation of growth plate cartilage in vivo. Proc Natl Acad Sci U S A 2004;101:14794–14799. [PubMed: 15459318]

Briegel KJ, Baldwin HS, Epstein JA, Joyner AL. Congenital heart disease reminiscent of partial trisomy 2p syndrome in mice transgenic for the transcription factor Lbh. Development 2005;132:3305–3316. [PubMed: 15958514]

Briegel KJ, Joyner AL. Identification and characterization of Lbh, a novel conserved nuclear protein expressed during early limb and heart development. Dev Biol 2001;233:291–304. [PubMed: 11336496]

Carlevaro MF, Cermelli S, Cancedda R, Descalzi Cancedda F. Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci 2000;113(Pt 1):59–69. [PubMed: 10591625]

Chung UI, Lanske B, Lee K, Li E, Kronenberg H. The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. Proc Natl Acad Sci U S A 1998;95:13030–13035. [PubMed: 9789035]

Deckers MM, Karperien M, van der Bent C, Yamashita T, Papapoulos SE, Lowik CW. Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. Endocrinology 2000;141:1667–1674. [PubMed: 10803575]

Dong YF, Soung do Y, Schwarz EM, O'Keefe RJ, Drissi H. Wnt induction of chondrocyte hypertrophy through the Runx2 transcription factor. J Cell Physiol 2006;208:77–86. [PubMed: 16575901]

Engsig MT, Chen QJ, Vu TH, Pedersen AC, Therkidsen B, Lund LR, Henriksen K, Lenhard T, Foged NT, Werb Z, Delaisse JM. Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. J Cell Biol 2000;151:879–889. [PubMed: 11076971]

Enomoto H, Enomoto-Iwamoto M, Iwamoto M, Nomura S, Himeno M, Kitamura Y, Kishimoto T, Komori T. Cbfa1 is a positive regulatory factor in chondrocyte maturation. J Biol Chem 2000;275:8695–8702. [PubMed: 10722711]

Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R. Toward a molecular understanding of skeletal development. Cell 1995;80:371–378. [PubMed: 7859279]

Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 1999;5:623–628. [PubMed: 10371499]

Grimsrud CD, Romano PR, D'Souza M, Puzas JE, Reynolds PR, Rosier RN, O'Keefe RJ. BMP-6 is an autocrine stimulator of chondrocyte differentiation. J Bone Miner Res 1999;14:475–482. [PubMed: 10234567]

Hahm GK, Barth RF, Schauer GM, Reiss R, Opitz JM. Trisomy 2p syndrome: a fetus with anencephaly and postaxial polydactyly. Am J Med Genet 1999;87:45–48. [PubMed: 10528246]

Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, Himeno M, Sato M, Yamagiwa H, Kimura T, Yasui N, Ochi T, Endo N, Kitamura Y, Kishimoto T, Komori T. Maturational disturbance of chondrocytes in Cbfa1-deficient mice. Dev Dyn 1999;214:279–290. [PubMed: 10213384]

Karsenty G. Minireview: transcriptional control of osteoblast differentiation. Endocrinology 2001;142:2731–2733. [PubMed: 11415989]

Kim IS, Otto F, Zabel B, Mundlos S. Regulation of chondrocyte differentiation by Cbfa1. Mech Dev 1999;80:159–170. [PubMed: 10072783]

Komori T. Requisite roles of Runx2 and Cbfb in skeletal development. J Bone Miner Metab 2003;21:193–197. [PubMed: 12811622]

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997;89:755–764. [PubMed: 9182763]

Kronenberg HM. Developmental regulation of the growth plate. Nature 2003;423:332–336. [PubMed: 12748651]

Logan M, Tabin C. Targeted gene misexpression in chick limb buds using avian replication-competent retroviruses. Methods 1998;14:407–420. [PubMed: 9608511]

Maes C, Carmeliet P, Moermans K, Stockmans I, Smets N, Collen D, Bouillon R, Carmeliet G. Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Mech Dev 2002;111:61–73. [PubMed: 11804779]

Murtaugh LC, Chyung JH, Lassar AB. Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. Genes Dev 1999;13:225–237. [PubMed: 9925646]

Murtaugh LC, Zeng L, Chyung JH, Lassar AB. The chick transcriptional repressor Nkx3.2 acts downstream of Shh to promote BMP-dependent axial chondrogenesis. Dev Cell 2001;1:411–422. [PubMed: 11702952]

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 1997;89:765–771. [PubMed: 9182764]

Papachristou DJ, Papachristou GI, Papaefthimiou OA, Agnantis NJ, Basdra EK, Papavassiliou AG. The MAPK-AP-1/-Runx2 signalling axes are implicated in chondrosarcoma pathobiology either independently or via up-regulation of VEGF. Histopathology 2005;47:565–574. [PubMed: 16324193]

Sundin OH, Busse HG, Rogers MB, Gudas LJ, Eichele G. Region-specific expression in early chick and mouse embryos of Ghox-lab and Hox 1.6, vertebrate homeobox-containing genes related to Drosophila labial. Development 1990;108:47–58. [PubMed: 1693558]

Usui M, Xing L, Drissi H, Zuscik M, O'Keefe R, Chen D, Boyce BF. Murine and chicken chondrocytes regulate osteoclastogenesis by producing RANKL in response to BMP2. J Bone Miner Res 2008;23:314–325. [PubMed: 17967138]

Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell 1998;93:411–422. [PubMed: 9590175]

Zelzer E, Glotzer DJ, Hartmann C, Thomas D, Fukai N, Soker S, Olsen BR. Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. Mech Dev 2001;106:97–106. [PubMed: 11472838]

Zelzer E, Mamluk R, Ferrara N, Johnson RS, Schipani E, Olsen BR. VEGFA is necessary for chondrocyte survival during bone development. Development 2004;131:2161–2171. [PubMed: 15073147]

Zelzer E, McLean W, Ng YS, Fukai N, Reginato AM, Lovejoy S, D'Amore PA, Olsen BR. Skeletal defects in VEGF(120/120) mice reveal multiple roles for VEGF in skeletogenesis. Development 2002;129:1893–1904. [PubMed: 11934855]

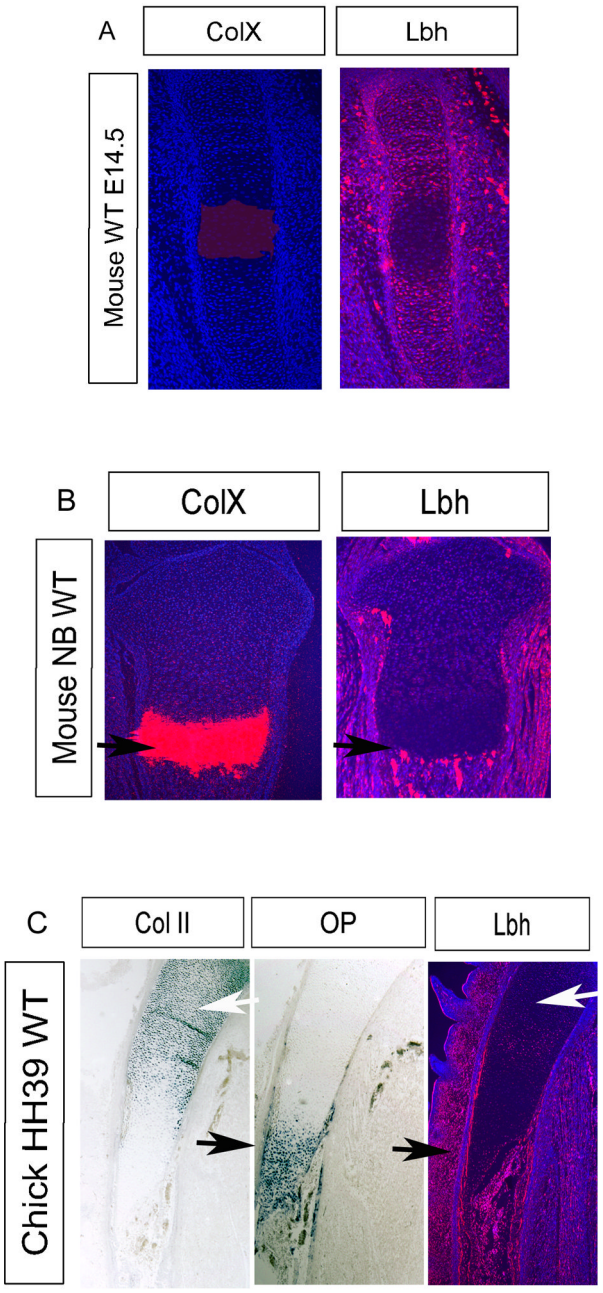

Figure 1.

Lbh expression in the fetal growth plate of mouse and chicken. **(A)** In WT mouse growth plates at E14.5 Lbh is highly expressed in round and flat proliferating chondrocytes while there is no Lbh expression in early hypertrophic chondrocytes (compared to collagen X mRNA (ColX;Col10a) expression). Lbh is also expressed in cells of the perichondrium and in the surrounding soft tissues. **(B)** ISH for Lbh in WT NB mice, showing that Lbh is expressed in round, flat and late hypertrophic chondrocytes, cells of the POC, the perichondrium and in the bone surrounding tissues. (black arrows indicate identical positions near lower end of region of collagen X mRNA expression on serial sections of the same bone.) **(C)** ISH for Lbh in WT chickens at stage HH39 (E11). Lbh is expressed in round (white arrows, compare to col II

mRNA expression on a serial section of the same bone), flat, and late hypertrophic chondrocytes (black arrows, compare to OP mRNA expression on a serial section of the same bone), cells of the POC, the perichondrium and in the tissues surrounding bone.

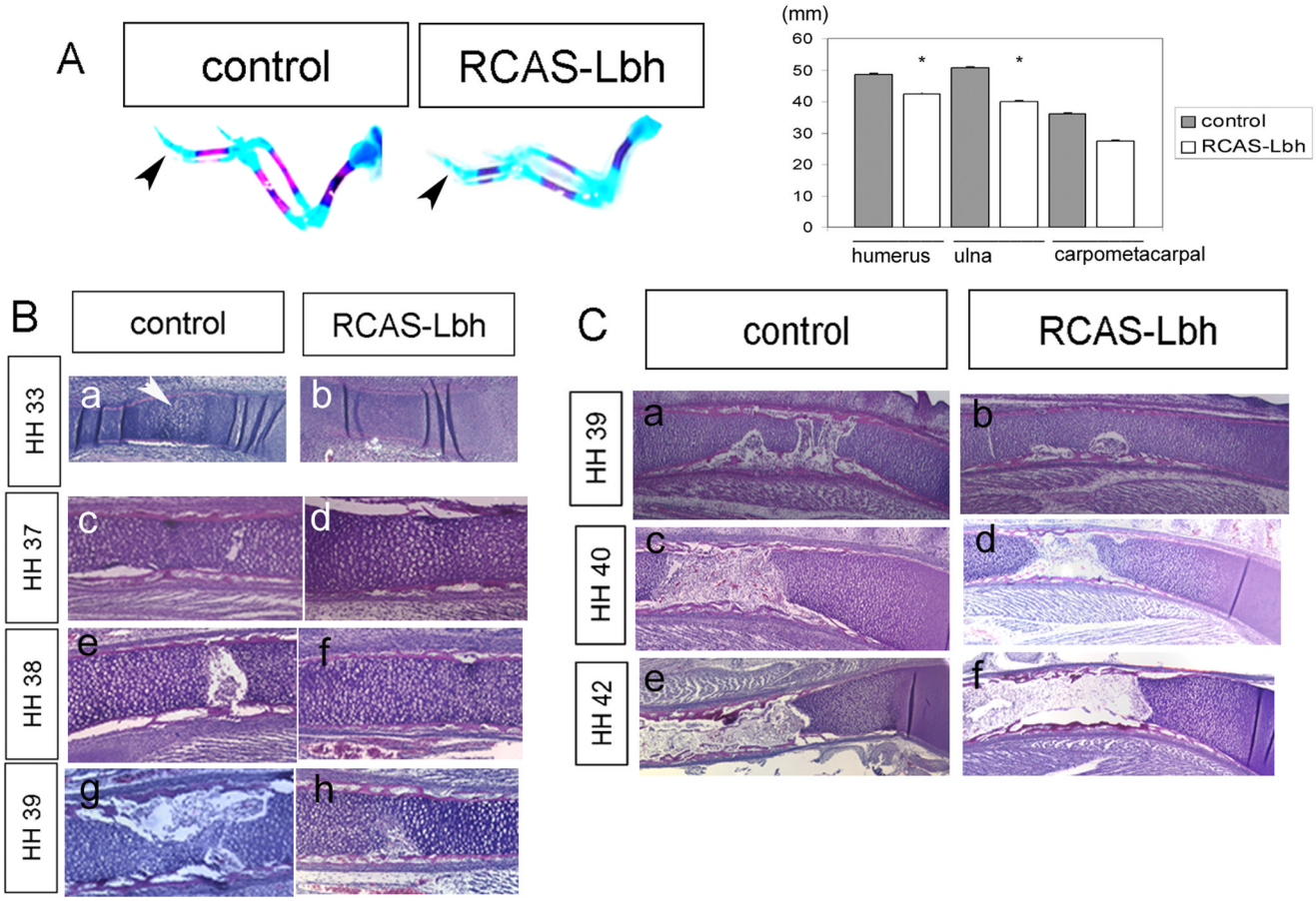

Figure 2.

RCAS-Lbh overexpression delays chondrocyte hypertrophy, vascular invasion and the formation of the POC. **(A)** Skeletal preparations stained with alcian blue and alizarin red at HH39 show a shortened RCAS-Lbh infected wing. Additionally, the zone of mineralization appears shorter and occurs in the first digit of the control wing while it is still missing in the Lbh overexpressing first digit (arrowheads). Quantification of the humerus, ulna and carpometacarpal length demonstrates a significant reduction of the infected bones. Values are expressed as means ±SEM. *P<0.01. **(B) (a+b)** H&E staining at stage HH33 (ulnae, u) shows a delay in hypertrophic chondrocytes of infected wings (white arrowhead points to hypertrophic chondrocytes). **(c–h)** H&E staining between HH37 and HH39 (carpometacarpal, CMC) shows a delay of vascular invasion and bone formation of infected wings. **(C) (a–d)** H&E staining between HH39 and HH40 shows a delay of vascular invasion and bone formation of infected wings. **(e+f)** This phenotype catches up over time (HH42).

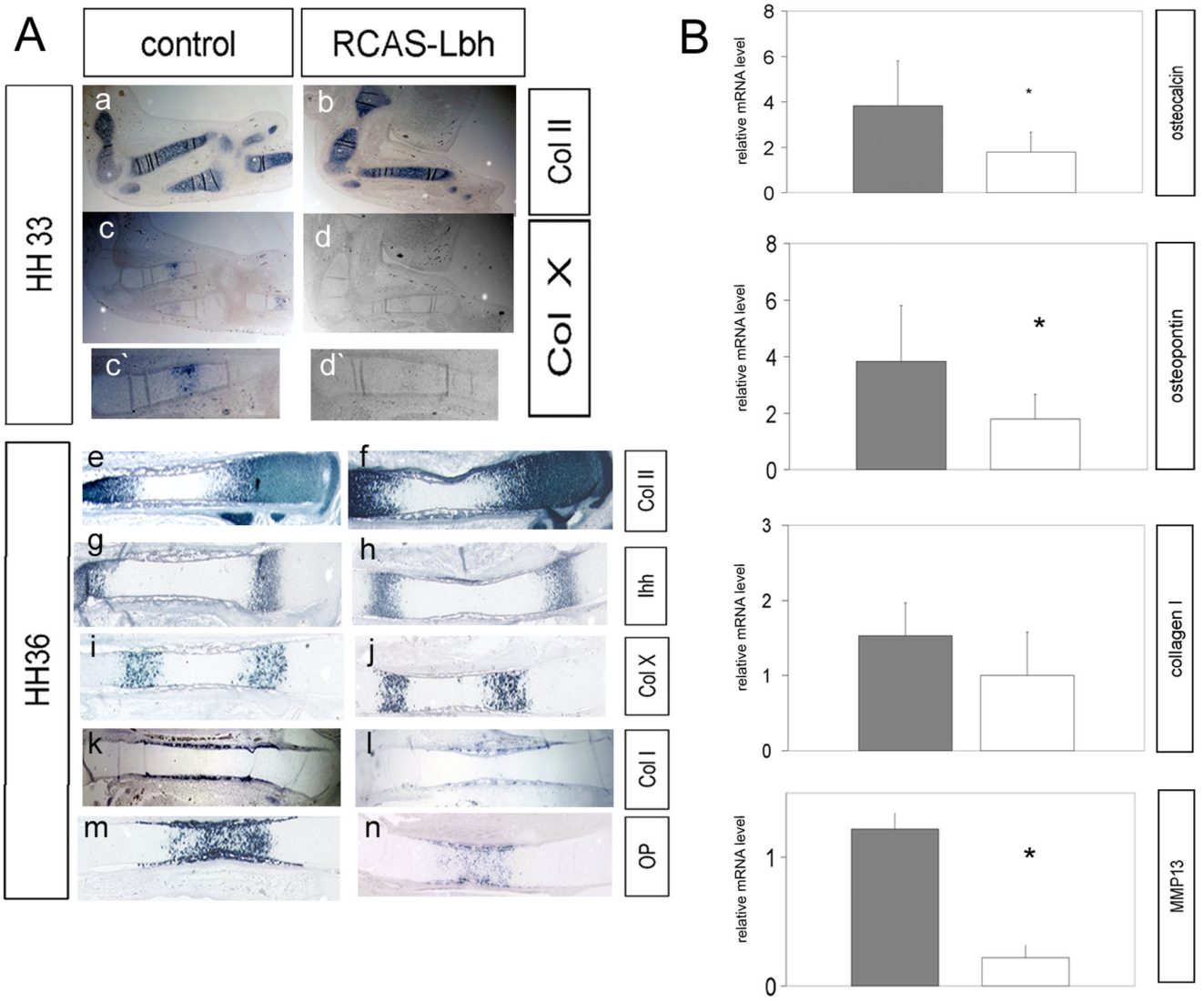

Figure 3.

Molecular analysis of RCAS-Lbh overexpressing bones. **(A) (a–d)** ISH analyses for col II and col X mRNA at HH33. The pattern of col II transcripts is similar in both control and RCAS-Lbh overexpressing bones whereas col X expression is delayed in RCAS-Lbh overexpressing bones. **(c`+d`)** higher magnifications of c and d. **(e–h)** ISH analyzes for genes expressed in proliferating chondrocytes (**(e, f)** coll II mRNA) and prehypertrophic chondrocytes, (**(g, h)** Ihh mRNA) at HH36 revealed a comparable mRNA expression pattern in both control and RCAS-Lbh overexpressing CMC. **(i+j)** ISH for col X at HH36 CMC shows a similar expression pattern in control and Lbh-overexpressing bones, reflecting a catch up of the phenotype seen earlier. **(k+l)** ISH for col I mRNA expression at HH36 shows col I expression in control CMC in the perichondrial region of the developing bones, while Lbh-overexpressing CMC shows weak expression in the perichondrial area. **(m+n)** ISH for OP mRNA appeared with a weaker and shorter expression area in Lbh-overexpressing bones compared to the control bones. **(B)** QRT-PCR analyses of genes related to osteoblast and late hypertrophic chondrocyte differentiation in HH39 old control (gray bars) and RCAS-Lbh (white bars) overexpressing wings (n=10). Expression of OC, OP, Col I and MMP13 mRNA is decreased in RCAS-Lbh overexpressing

bones as compared to the control. Values are expressed as means ±SEM and represent the relative mRNA expression level normalized to GAPDH expression level. *p<0.01.

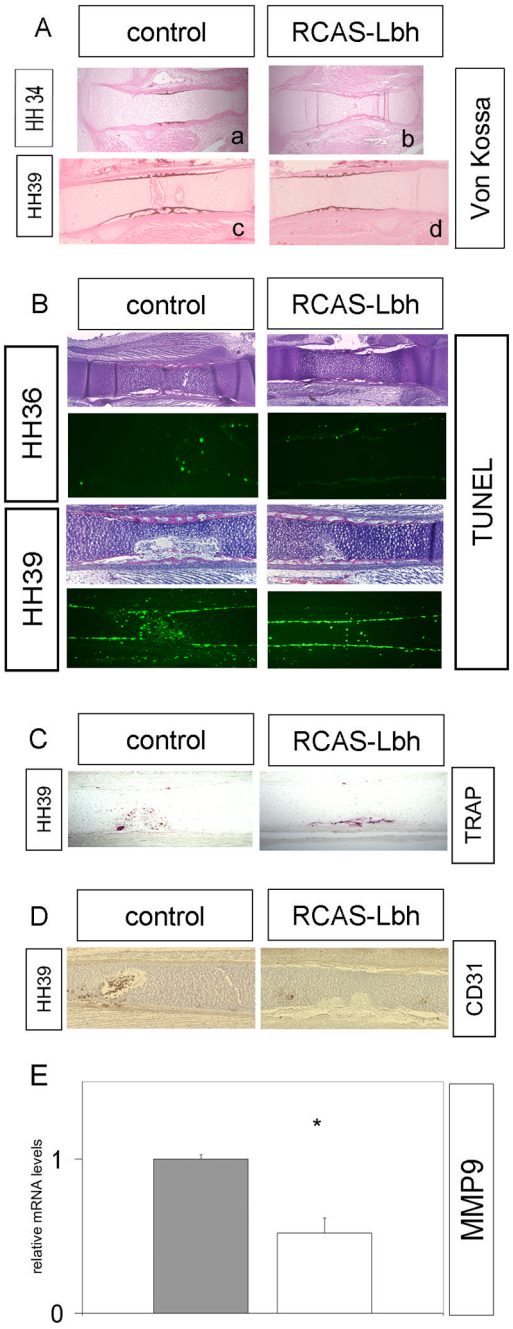

Figure 4.

Effect of RCAS-Lbh overexpression on bone mineralization, cartilage resorption and osteoclast and endothelial cell invasion. **(A) (a+b)** Von Kossa (v.K.) staining of the CMC at HH36 as well as HH39 **(c+d)** illustrating impaired bone collar mineralization in RCAS-Lbh overexpressing bones compared to control bones. **(B)** TUNEL staining of CMC of HH36 and HH39 old animals. H&E stained serial sections are included to identify the regions of cellular apoptosis. While control CMC show apoptotic cells in the region of vascular invasion, invasion itself as well as apoptosis are delayed in the RCAS-Lbh overexpressing CMC. **(C)** TRAP stained CMC at HH39 reveal TRAP positive cells in the centers of the midshafts in control bones, whereas in the RCAS-Lbh overexpressing bones, the TRAP positive cells occur in the

region of the perichondrium. **(D)** IHC for CD31 at HH39 CMC. Like in the TRAP staining, brown CD31 staining is more intense and occurs in the center of the bones' midshafts where vascular invasion occurs in the control CMC. The Lbh overexpressing bone shows some single CD31-stained brown cells in the perichondrial region. **(E)** QRT-PCR analyzes of the MMP9 gene related to cartilage resorption and osteoclast invasion in HH39 old control (gray bars) and RCAS-Lbh (white bars) overexpressing wings (n=10). Expression of MMP9 mRNA is decreased in RCAS-Lbh overexpressing bones as compared to control bones. Values are expressed as means ±SEM and represent the relative mRNA expression level determined as the ratio of the respective signal to the signal obtained for the chicken GAPDH gene. *$p<0.02$.

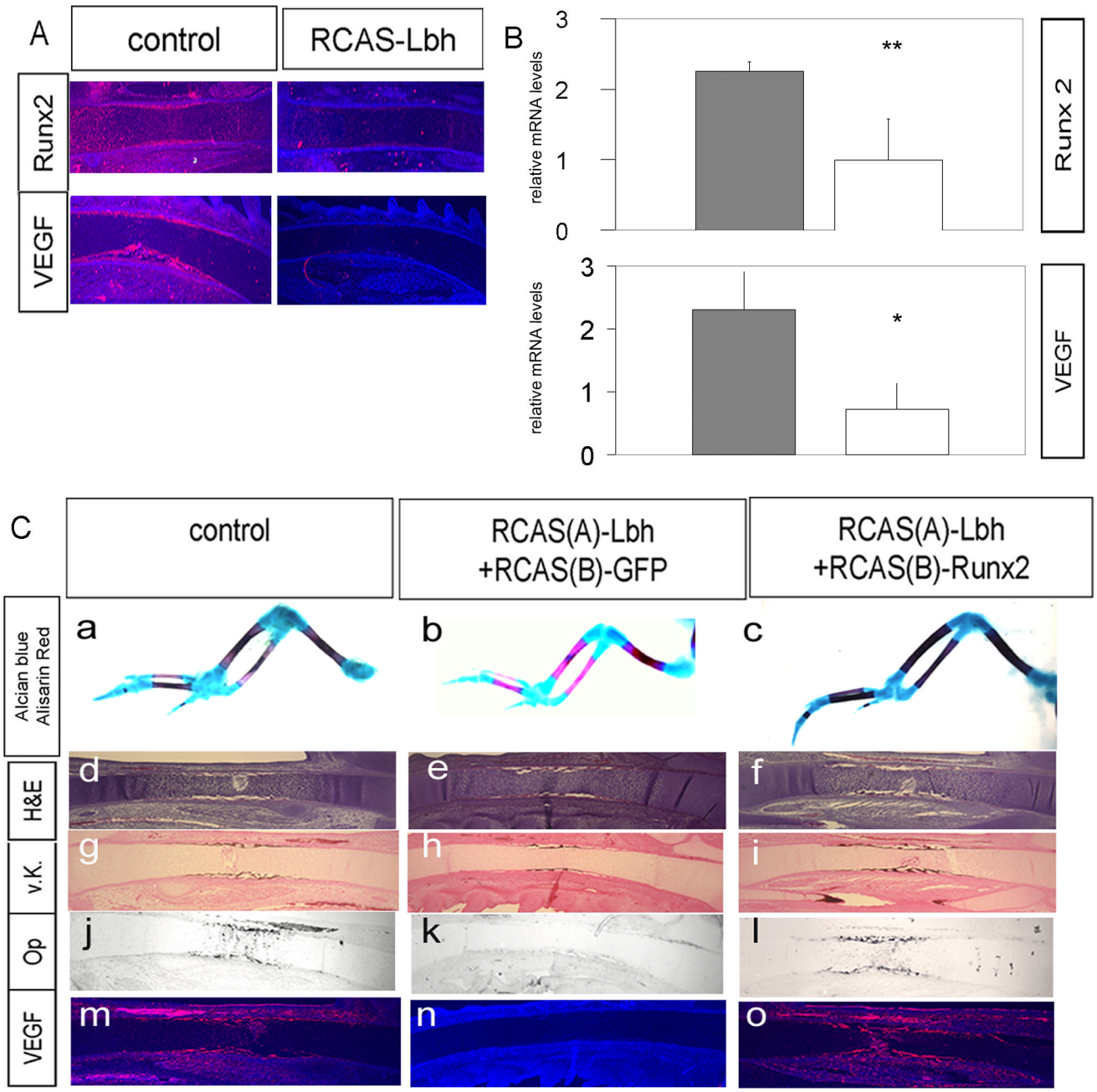

Figure 5.

RCAS-Lbh overexpression represses Runx2 and VEGF mRNA which can be rescued by co-overexpressing Lbh and Runx2 **(A)** ISH at HH36 (CMC) shows a dramatic repression of Runx2 and VEGF transcripts. **(B)** QRT-PCR analyses of Runx2 and VEGF mRNA expression in stage HH39 old control (gray bars) and RCAS-Lbh (white bars) overexpressing wings (n=10). Expression of both Runx2 and VEGF mRNA is decreased in RCAS-Lbh overexpressing bones as compared to control bones. Values are expressed as means ±SEM and represent the relative mRNA expression level determined as the ratio of the respective signal to the signal obtained for the chicken GAPDH gene. *p<0.02, **p<0.05. **(C)** Co-overexpression of RCAS(A)-Lbh (Lbh) and RCAS(B)-Runx2 (Runx2) or Lbh and RCAS-(B)-GFP (GFP) were performed at

stage HH20 and reincubated until HH39. Serial sections from CMC are shown. **(a–c)** In Lbh-Runx2 double infected wings skeletal preparations show a rescue of the shortened skeletal elements seen in the Lbh-GFP infected wing in comparison to the control wing. **(d–i)** In Lbh-Runx2 double infected wings H&E and von Kossa staining shows a rescue of the delayed vascular invasion and bone collar formation seen in the Lbh-GFP infected wing in comparison to the control wing.**(j–o)** In Lbh-Runx2 double infected wings, in ISH for OP and VEGF shows a rescue of the dramatic downregulation of both markers in the Lbh-GFP infected versus the control wing.

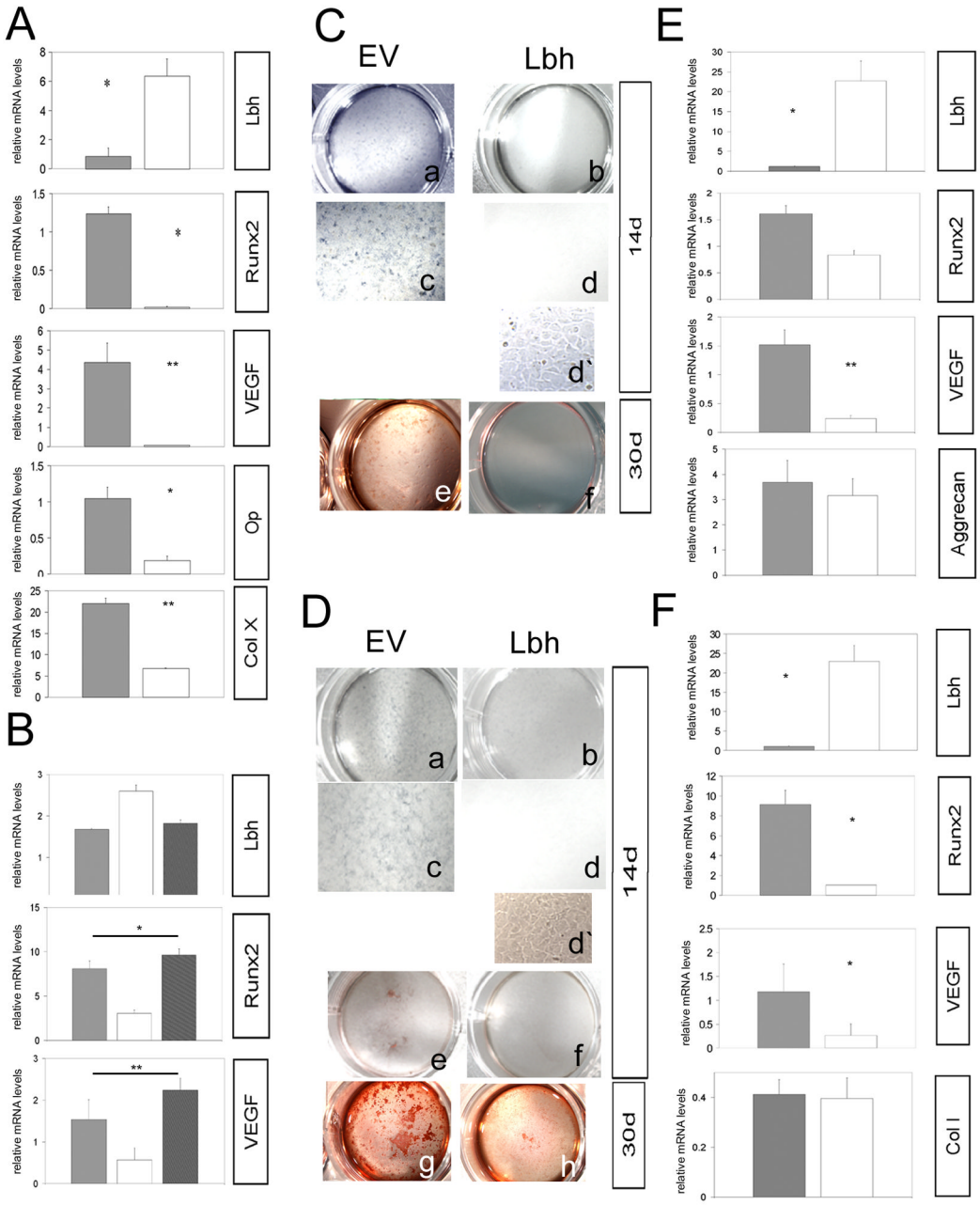

Figure 6.

Forced Lbh expression *in vitro* in avian **(A+B)** and murine **(C–F)** cell lines. **(A)** Lbh overexpression in primary cultures of chicken chondrocytes decreases mRNA levels of Runx2, VEGF, OP and col X. Upper sternal chondrocytes were infected with RCAS(B)-Lbh retrovirus (white bar) or RCAS(B)-empty vector (EV) (gray bar) and cultured for 7 d. Gene expression was assayed by QRT-PCR. Values are expressed as means ±SEM and represent the relative mRNA expression level determined as the ratio of the respective signal to the signal obtained for the chicken GAPDH gene. *p<0.01, **p<0.001. **(B)** Lbh and Runx2 co-overexpression in upper sternal chondrocytes can rescue the decreased levels of Runx2 and VEGF mRNA. Upper sternal chondrocytes were infected with RCAS(B)-EV (gray bar), RCAS(B)-Lbh (white bar)

or RCAS(B)-Lbh/RCAS(A)-Runx2 (black bar) retrovirus and cultured for 7 d. Gene expression was assayed by QRT-PCR. Values are expressed as means ±SEM and represent the relative mRNA expression level determined as the ratio of the respective signal to the signal obtained for the chicken GAPDH gene. *p<0.02, **p<0.01. **(C)** Forced Lbh expression in ATDC5 cells delays alkaline phosphatase (AP) expression and formation of mineralization nodules. ATDC5 cells were stably transfected with pcDNA3.1 empty vector (EV) or pcDNA3.1-Lbh vector (Lbh). **(a–d)** AP staining at 14d. **(d`)** phase contrast picture of d. **(e+f)** Alizarin Red staining at 30d. **(D)** Forced Lbh expression in MC3T3 cells delays alkaline phosphatase (AP) expression and formation of mineralization nodules. MC3T3 cells were stably transfected with pcDNA3.1 empty vector (EV) or pcDNA3.1-Lbh vector (Lbh). **(a–d)** AP staining at 14d. **(d`)** phase contrast picture of d. **(e+f)** Alizarin Red staining at 14d. **(g+h)** Alizarin red staining at 30d. **(E)** Lbh missexpression in ATDC5 cells decreases mRNA levels of Runx2 and VEGF. ATDC5 cells were stably transfected with pcDNA3.1 EV (gray bar) or pcDNA3.1 Lbh (white bar) and cultured for 7 d. Gene expression was assayed by QRT-PCR. Values are expressed as means ±SEM and represent the relative mRNA expression level determined as the ratio of the respective signal to the signal obtained for the mouse β-actin gene. *p<0.01, **p<0.004. **(F)** Lbh missexpression in MC3T3 cells decreases mRNA levels of Runx2 and VEGF. MC3T3 cells were stably transfected with pcDNA3.1 EV (gray bar) or pcDNA3.1 Lbh (white bar) and cultured for 7 d. Gene expression was assayed by QRT-PCR. Values are expressed as means ±SEM. *p<0.01.