Deletion of *Vhlh* in chondrocytes reduces cell proliferation and increases matrix deposition during growth plate development

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Summary

The von Hippel Lindau tumor suppressor protein (pVHL) is a component of a ubiquitin ligase that promotes proteolysis of the transcription factor hypoxia-inducible-factor 1α (HIF1α), the key molecule in the hypoxic response. We have used conditional inactivation of murine *VHL (Vhlh)* in all cartilaginous elements to investigate its role in endochondral bone development. Mice lacking *Vhlh* in cartilage are viable, but grow slower than control littermates and develop a severe dwarfism. Morphologically, *Vhlh* null growth plates display a significantly reduced chondrocyte proliferation rate, increased extracellular matrix, and presence of atypical large cells within the resting zone. Furthermore, stabilization of the transcription factor HIF1α leads to increased expression levels of HIF1α target genes in *Vhlh* null growth plates. Lastly, newborns lacking both *Vhlh* and *Hif1a* genes in growth plate chondrocytes display essentially the same phenotype as *Hif1a* null single mutant mice suggesting that the *Vhlh* null phenotype could result, at least in part, from increased activity of accumulated HIF1α. This is the first study reporting the novel and intriguing findings that pVHL has a crucial role in endochondral bone development and is necessary for normal chondrocyte proliferation in vivo.

Key words: von Hippel-Lindau tumor suppressor protein, Cartilage development, HIF1α

Introduction

Skeletal development depends on two mechanisms: intramembranous and endochondral bone formation (Erlebacher et al., 1995). The former, in which mesenchymal cells develop directly into osteoblasts, is involved in the formation of the flat skull bones. The latter, accounting for the development of most other bones, involves a two-stage mechanism whereby chondrocytes form a matrix template, the growth plate, in which osteoblasts differentiate and initiate the ossification process. An understanding of this process at the molecular level is emerging (Kronenberg, 2003; Ferrara et al., 2003; Ortega et al., 2003; Olsen et al., 2000; Karsenty, 1999; Harper and Klagsbrun, 1999; Erlebacher et al., 1995). During endochondral bone development, growth plate chondrocytes undergo well-ordered and controlled phases of cell proliferation, differentiation and apoptosis. Round proliferative chondrocytes synthesize type II collagen and form a columnar layer, and then differentiate into postmitotic hypertrophic cells, which express predominantly type X collagen; these cells also synthesize vascular endothelial growth factor A (VEGF) (Kirsch and von der Mark, 1991; Gerber et al., 1999). This unique differentiation process is followed by death of hypertrophic chondrocytes, blood vessel invasion, and finally replacement of the cartilaginous matrix with trabecular bone (Hatori et al., 1995).

The fetal growth plate is a constitutively avascular tissue, and we have recently demonstrated that it is hypoxic, with a typical outside-inside gradient of oxygenation (Schipani et al., 2001). Adaptation of cells and tissues to hypoxic microenvironments requires the presence of hypoxia-inducible factor 1α (HIF1α), which is the key molecule in hypoxic response, regulating the expression of glycolytic enzymes and several growth factors, including VEGF (Semenza, 2000). This transcription factor is ubiquitously expressed, but under normoxic conditions it is hydroxylated on specific proline residues by a recently discovered family of prolyl-hydroxylases (Jaakkola et al., 2001). The von Hippel Lindau tumor suppressor protein (pVHL), which is a recently identified novel E3 ubiquitin ligase (Iliopoulos et al., 1995), recognizes the proline-hydroxylated form of HIF1α, and targets it for polyubiquitination and degradation by the proteasome. Conversely, under hypoxic conditions, oxygen-sensitive prolyl-hydroxylase activity is reduced, and HIF1α translocates into the nucleus. Within the nucleus it binds to its

putative partner HIF1β, also termed ARNT (aryl hydrogen receptor nuclear translocator). This complex binds to specific hypoxic responsive elements (HRE), thereby initiating the transcription of specific genes (Forsythe et al., 1996).

pVHL consists of 213 amino acids, and is expressed in most tissues and cell types (Iliopoulos et al., 1995). Heterozygous missense mutations of the *VHL* gene have been identified as the likely cause of the von Hippel Lindau syndrome (Latif et al., 1993). This syndrome is characterized by a dominantly inherited predisposition to develop pheochromocytomas, and highly vascular tumors of the kidney, the central nervous system and the retina (Maher and Kaelin, 1997). Tumorigenesis is associated with either the loss or inactivation of the wild-type allele, following Knudson's two hit hypothesis (Knudson and Meadows, 1980). pVHL has been shown to form a stable multiprotein complex which contains Elongin B (TCEB2), Elongin C (TCEB1), CUL2 and RBX1 (Kibel et al., 1995; Ohh et al., 2000). As mentioned above, this multiprotein complex has E3 ubiquitin-ligase activity, and one of its main targets is HIF1α (Lisztwan et al., 1999; Iwai et al., 1999). The importance of pVHL for proteolysis of HIF1α is further underlined by the finding that cells lacking a functional pVHL are unable to degrade this transcription factor, which ultimately results in an accumulation of HIF1α. Homozygous disruption of *Vhlh* in mice results in early embryonic lethality caused by abnormalities of placental vasculogenesis (Gnarra et al., 1997). Thus, little is known about the physiological role of pVHL during fetal development and postnatal life.

We have recently reported that HIF1α is essential for cell growth and survival of murine growth plate chondrocytes in vivo (Schipani et al., 2001). Chondrocytes lacking functional HIF1α undergo massive cell death in the center of the growth plate (Schipani et al., 2001). Interestingly, viable chondrocytes at the periphery display a significant increased rate of actively proliferating cells. Furthermore, we have provided clear evidence that HIF1α is necessary for type II collagen accumulation by hypoxic growth plate chondrocytes in vitro (Pfander et al., 2003). Based on these findings, we hypothesized pVHL, a key molecule in the degradation pathway of HIF1α, to be an important modulator of long bone development, possibly through the regulation of HIF1α stability. The aims of this study were to explore the role of pVHL in endochondral bone development, and to investigate whether the lack of pVHL might affect expression of HIF1α target genes in growth plate chondrocytes.

Materials and methods

Generation of conditional *Vhlh* and double knock out

Hemizygous transgenic mice in which Cre recombinase expression was driven by the *Col2a1* promoter (Schipani et al., 2001) were bred with mice homozygous for a $Vhlh^{+f}$ allele (Haase et al., 2001), and/or with mice homozygous for a Hif^{+f} allele (Schipani et al., 2001). After appropriate breeding, $Vhlh^{+f/+f}$*;Col2a1Cre* and $Vhlh^{+f/+f}$ $Hif^{+f/+f}$*;Col2a1Cre* mutant mice were generated. Genotyping was performed as previously described (Schipani et al., 2001; Haase et al., 2001).

Alizarin Red S staining, histological and in situ hybridization analyses, Tunel-assay

Alizarin Red S staining was performed as described previously (Schipani et al., 1997).

For light microscopy, tissues from E14.5, E15.5, E16.5, E17.5 and E18.5 embryos (delivered by caesarean section), from newborns, and from 5- and 30-day-old mice, were fixed in 10% formalin/PBS (pH 7.4) and stored in fixative at 4°C. Paraffin blocks were prepared by standard histological procedures. Sections (5-6 μm in thickness) were cut from several levels of the block, and stained with Hematoxylin (H) and Eosin (E). In situ hybridization was performed using complementary ^{35}S-labeled riboprobes as described previously (Lee et al., 1996).

For Tunel assay, paraffin sections from hindlimbs of newborn mice were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Tunel assay was performed using the In Situ Cell Death Detection Kit (Roche, Germany), according to the manufacturer's instructions.

Bromodeoyxyuridine (BrdU) labeling

E14.5, E15.5 and E16.5 pregnant mice were injected intraperitoneally with 100 μg BrdU/12 μg FdU per gram body weight, 2 hours prior to sacrifice. After sacrifice, embryo hindlimbs were dissected, fixed and embedded in paraffin, and longitudinal sections across the tibia and femur were obtained. To identify actively proliferating cells, nuclei that had incorporated BrdU were detected using a Zymed BrdU immunostaining kit (Zymed Laboratories, San Francisco, USA).

HIF1α immunohistochemistry

For HIF1α detection, fresh frozen sections from newborn mice were fixed in acetone for 20 minutes at –20°C and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After blocking, sections were incubated with the commercially available antibody C-19 that specifically recognizes an epitope in the C-terminal portion of the HIF1α protein (Santa Cruz Biotech, CA, USA), at a dilution of 1:100. Detection of binding was by the Streptdavidin-HRP system provided by the TSA kit (Perkin Elmer-Life Sciences, MA, USA), according to the manufacturer's conditions.

Chondrocyte isolation, western blot analysis, real-time PCR, Elisa

Chondrocytes were isolated from newborn $Vhlh^{+f//+f}$ mice as described (Pfander et al., 2003). In brief, forelimbs and hindlimbs were dissected, and distal epiphyses of radius, ulna and tibia were isolated in HBSS (Gibco BRL, MD, USA). Epiphyses were digested in 0.25% trypsin/EDTA for 30 minutes at 37°C, and in 195 U/ml collagenase type II in HBSS (Worthington, NJ, USA). Chondrocytes were plated at a density of 4×10^5 cells per well of a six-well plate, and grown in monolayer cultures in high glucose DMEM (Gibco BRL, MD, USA) supplemented with 10% FBS (Hyclone, UT, USA) and 1% penicillin/streptomycin. On day 1 post-plating, adherent chondrocytes were infected with adenovirus containing either β-galactosidase or Cre recombinase (generously supplied by Frank J. Giordano, Yale University, New Haven, CT, USA) to create wild-type chondrocytes or *Vhlh* null cells. Deletion of *Vhlh* was confirmed by semi-quantitative PCR analysis across the 3′ loxP site. For immunoblotting, cells were lyzed, and detection of HIF1α hydroxylated at Pro564 was performed as previously described (Chan et al., 2002). Detection of α-tubulin with a specific antibody (Research Diagnostics, NJ, USA) was used as control of equal loading.

Total RNA was isolated from wild-type chondrocytes or *Vhlh* null cells as previously described (Pfander et al., 2003), and then transcribed into single cDNA using AMV reverse transcriptase (Boehringer Mannheim, Germany) and random hexamer primers, according to the manufacturer's instructions. For real-time PCR analyses, cDNA was diluted to a final concentration of 10 ng/μl. For PCR reactions, SYBR-Green Mastermix (Applied Biosytems) was used. Total cDNA (50 ng) was used as template to determine the relative amount of mRNA by real-time PCR (ABI Prism 7700 sequence detection system) using specific primers. The reaction was conducted as follows: 95°C for 4 minutes, and then 40 cycles of 15 seconds 95°C and 1 minute 60°C. β-actin was amplified as an internal

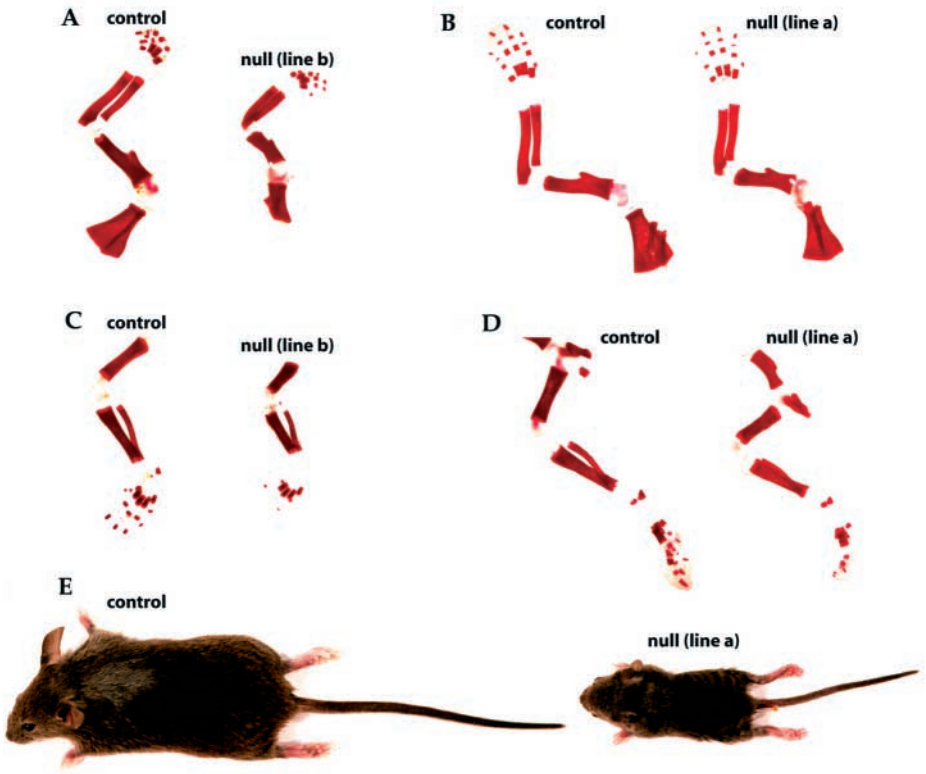

Fig. 1. Alizarin Red S staining of forelimbs and hindlimbs of E18.5 control and *Vhlh* null (*Cre* transgenic line b) mice (A,C), and of newborn control and *Vhlh* null (*Cre* transgenic line a) mice (B,D). (E) Four-week-old control and *Vhlh* null mice.

control. Cycle threshold (Ct) values were measured and calculated by the Sequence detector software. Relative amounts of mRNA were normalized to β-actin and calculated using the software program Microsoft Excel. Relative mRNA contents were calculated as $x=2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct=\Delta E-\Delta C$, $\Delta E=Ct_{VHL\ null}-Ct_{\beta\text{-actin}}$ and $\Delta C=Ct_{WT}-Ct_{\beta\text{-actin}}$ (wild-type expression was taken as 1). Specific primers for VEGF, phosphoglycerokinase 1 (PGK1) and type II collagen mRNA were designed as described (Pfander et al., 2003). For β-actin the following sequences were used: β-actin forward, AGG CCC AGA GCA AGA GAG G; and β-actin reverse, TAC ATG GCT GGG GTG TTG AA.

Protein concentrations of soluble VEGF isoforms were determined using the DuoSet Elisa Kit for mouse VEGF (R&D Systems, MN, USA) as previously described (Pfander et al., 2003). Briefly, cell culture supernatants (wild-type chondrocytes or *Vhlh* null cells) were harvested and stored at –20°C. VEGF Elisa was conducted according to the manufacturer's instructions.

Results

Loss of *Vhlh* in growth plate chondrocytes results in a severe dwarfism

Because of the early lethality of mice nullizygous for *Vhlh* (at approximately embryonic day 8.5) before skeletal structures are formed, we used conditional inactivation of *Vhlh* in all cartilaginous elements to investigate the role of this tumor suppressor gene in growth plate development and chondrocyte biology (Gnarra et al., 1997; Schipani et al., 2001). For this purpose, mice carrying the *Cre recombinase* transgene driven by the *Col2a1* promoter (Schipani et al., 2001) were bred with mice homozygous for the floxed *Vhlh* allele in order to generate $Vhlh^{+f/+f}$ *Col2a1Cre* animals (null). Both the $Vhlh^{+f/+f}$ and the *Col2a1Cre* mice had been previously used in conditional-knockout experiments (Schipani et al., 2001; Kobayashi et al., 2002; Haase et al., 2001; Cramer et al., 2003). In particular, the two previously reported independent transgenic lines *Col2a1Cre* a and b, in which expression of the transgene in cartilage had been confirmed by in situ hybridization analysis and by breeding with *LacZ* reporter mice (Schipani et al., 2001; Kobayashi et al., 2002), were used in this study. Qualitatively similar results were obtained with both transgenic lines. However, transgenic line b mice were modestly smaller than control littermates. Furthermore, *Vhlh* null animals generated with transgenic line a had better postnatal survival. Therefore, most of the studies were conducted with transgenic line a. $Vhlh^{+f/+f}$, $Vhlh^{+f/+}$*Col2a1Cre*, and $Vhlh^{+/+}$ *Col2a1Cre* mice were used as wild-type control animals. Null newborns lacking *Vhlh* in all cartilaginous elements were viable at birth. However, compared with their normal littermates, mutant newborns were overall smaller with a characteristic shortening of their limb skeleton (Fig. 1A-D). The degree and pattern of skeletal mineralization was similar in both control and mutant newborns, as shown by Alizarin red S staining (Fig. 1A-D). After birth *Vhlh* null mice grew slower than their normal littermates and displayed a severe dwarfism (Fig. 1E).

Vhlh null growth plate displays severe hypocellularity, increased matrix, and presence of 'atypical' cells

A cartilage phenotype was already evident in *Vhlh* mutant embryos starting from E14.5 (data not shown); the severity of this phenotype increased progressively in later stages of skeletal development (Fig. 2A-I). In mutant growth plates, a significantly reduced number of chondrocytes in both resting and proliferating zones was found, with an increased amount of extracellular matrix between the cells (Fig. 2D-I). Resting zone chondrocytes of normal littermates displayed the characteristic small round cell shape with a high nucleus to cytoplasm ratio (Fig. 2D). By contrast, the center of the resting zone of *Vhlh* null growth plate was mainly occupied by enlarged chondrocytes that resembled hypertrophic cells (Fig. 2E,F). This special phenotype, i.e. hypocellularity, presence of larger chondrocytes with a higher cytoplasm to nucleus ratio and an increased amount of extracellular matrix, was even more pronounced in long bones isolated from null mice generated with transgenic line b (Fig. 2C,F,I). In particular, the number of 'atypical' chondrocytes with an increased cytoplasm to nucleus ratio was significantly higher in these specimens. Furthermore, these cells appeared to be present in both the

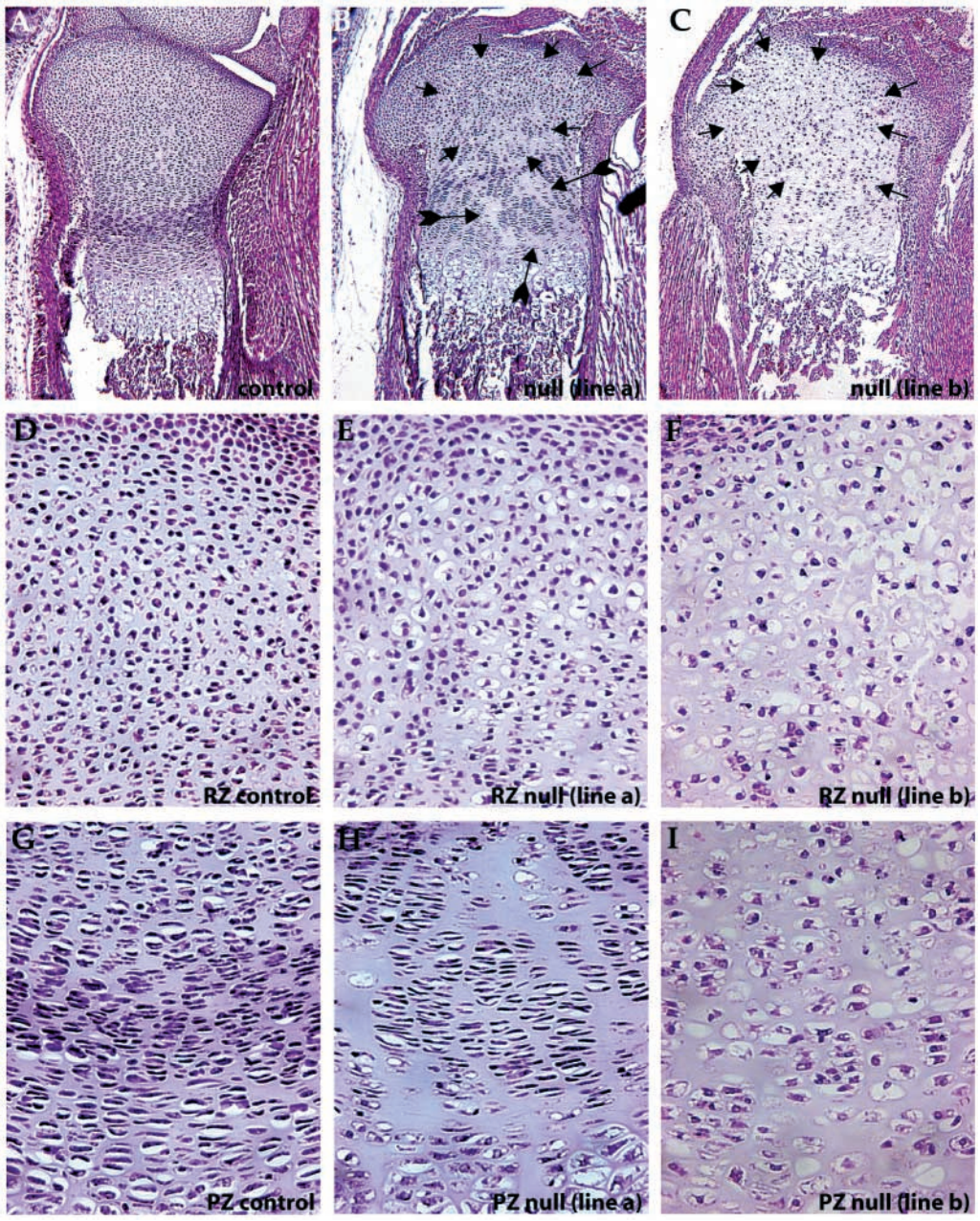

Fig. 2. Analysis of growth plates from control and *Vhlh* null mice with *Cre* transgenic lines a and b. Hematoxylin and Eosin staining of histological sections of control (A,D,G) and *Vhlh* null (B,C,E,F,H,I) tibia proximal growth plates at birth. (D-I) Magnification of the resting zone (D-F) and proliferative zone (G-I) of newborn control (D,G) and *Vhlh* null (E,F,H,I) growth plates. Arrowheads (B,C) indicate areas occupied with atypical enlarged chondrocytes; arrows indicate areas of increased matrix deposition within the *Vhlh* growth plate.

resting zone and in the columnar layer (Fig. 2F,I). Interestingly, the hypertrophic zone of *Vhlh* null mice was normally shaped without any obvious cellular or structural alterations; a modest and transient delay in blood vessel invasion and primary spongiosa formation could be detected in the mutant specimens at E14.5 and E15.5 (data not shown).

Chondocranium and axial cartilage of *Vhlh* null mice displayed a significantly milder phenotype than long bones (data not shown).

Lack of functional pVHL has been linked to both sporadic and familial cases of renal clear cell carcinomas, which are known to derive their histological appearance from accumulation of glycogen and lipids. We thus explored the possibility that the atypical chondrocytes of the *Vhlh* null epiphysis could have a ‘clear cell’ phenotype. Notably, PAS or Oil Red staining did not reveal any increased accumulation of glycogen or lipids, respectively, in the *Vhlh* null chondrocytes in comparison with control cells (data not shown).

To test whether the increased cell size of resting chondrocytes in *Vhlh* null animals might be the result of an ectopic or premature process of hypertrophic differentiation, type X collagen expression was investigated by in situ hybridization on histological sections of newborn mice. In both wild-type and mutant epiphyses, type X collagen mRNA expression was spatially restricted to the hypertrophic zone (Fig. 3C,G). Furthermore, a similar expression pattern of type II collagen mRNA was present in both *Vhlh* null and control growth plates (Fig. 3A,B,E,F). Consistent with these findings, spatial distribution of mRNA encoding the PTH/PTHrP receptor (PPR) and Indian Hedgehog (IHH), which are mainly

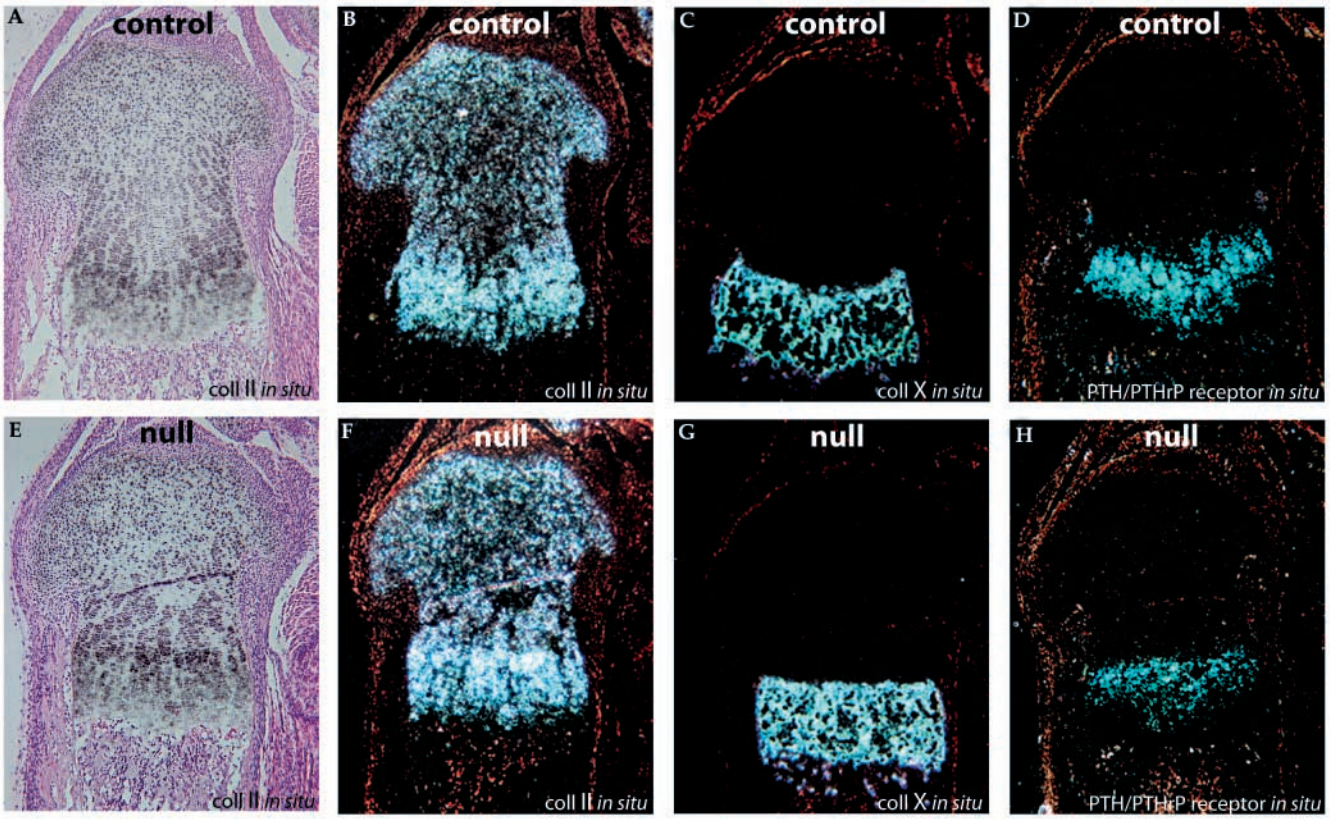

Fig. 3. In situ hybridization analysis of histological sections of proximal tibia growth plates from control (A-D) and *Vhlh* null (E-H) newborn mice, with type II collagen (A,B,E,F), type X collagen (C,G) and PTH/PTHrP receptor (D,H) cRNA; darkfield and brightfield images are shown.

expressed at the transition between proliferation and hypertrophy, was also similar in null and control specimens (Fig. 3D,H; and data not shown). Lastly, no differences in number and distribution of apoptotic chondrocytes were detected between mutant and control growth plates using the Tunel assay (data not shown). Taken together, our data indicate that the phenotypic alterations of resting chondrocytes in the *Vhlh* null growth plate were not the result of ectopic or premature hypertrophy.

Consistent with the increased amount of matrix in *Vhlh* null growth plates, as shown by histological analysis, real-time PCR of total RNA isolated from primary chondrocytes grown in normoxic conditions revealed an elevation of type II collagen mRNA expression in cells lacking *Vhlh* when compared with controls (Fig. 7C). This increase of collagen type II mRNA was not detectable by in situ hybridization analysis, probably as result of the severe hypocellularity of the mutant growth plate.

In the *Vhlh* null growth plate formation of the secondary ossification center is delayed

A few days after birth, the null growth plate was still smaller and hypocellular compared with the control (Fig. 4A,B). No blood vessel invasion could be identified in the areas where the secondary ossification center was being formed in wild-type growth plates (Fig. 4A,B). In situ hybridization analyses of 5-day-old control growth plates showed a typical expression pattern of PPR, IHH and type X collagen transcripts in the prehypertrophic-hypertrophic zone and in the area where the secondary ossification center was developing (Fig. 4E-J). These molecules are established markers of the hypertrophic differentiation process, which occurs at the primary and the secondary ossification centers. In accordance with the histological appearance, none of these markers could be detected in the corresponding area of the *Vhlh* null growth plate (Fig. 4E-J). It is also interesting to note that in the absence of *Vhlh*, the articular cartilage with its typical zonal architecture was greatly disturbed (Fig. 4C,D,K,L). Taken together, these findings strongly suggest that the lack of *Vhlh* delays the development of the secondary ossification center and the concomitant blood vessel invasion. Furthermore, loss of pVHL may also affect the formation of the hyaline articular cartilage.

Lack of *Vhlh* severely impairs chondrocyte proliferation

As no differences in apoptotic cell death were detected by Tunel analysis, we hypothesized that the reduced number of chondrocytes within the resting and proliferating zones of *Vhlh* null growth plates might result from a decreased mitotic activity. Therefore, we determined the number of chondrocytes in S-phase by BrdU labeling at different stages of fetal development. As shown in Fig. 5A,B, control and *Vhlh* null epiphyses displayed highly differing numbers of actively proliferating cells. Furthermore, by counting the percentage of BrdU-labeled cells, we established that, in the absence of *Vhlh*, chondrocytes proliferated at a significantly lower rate both in

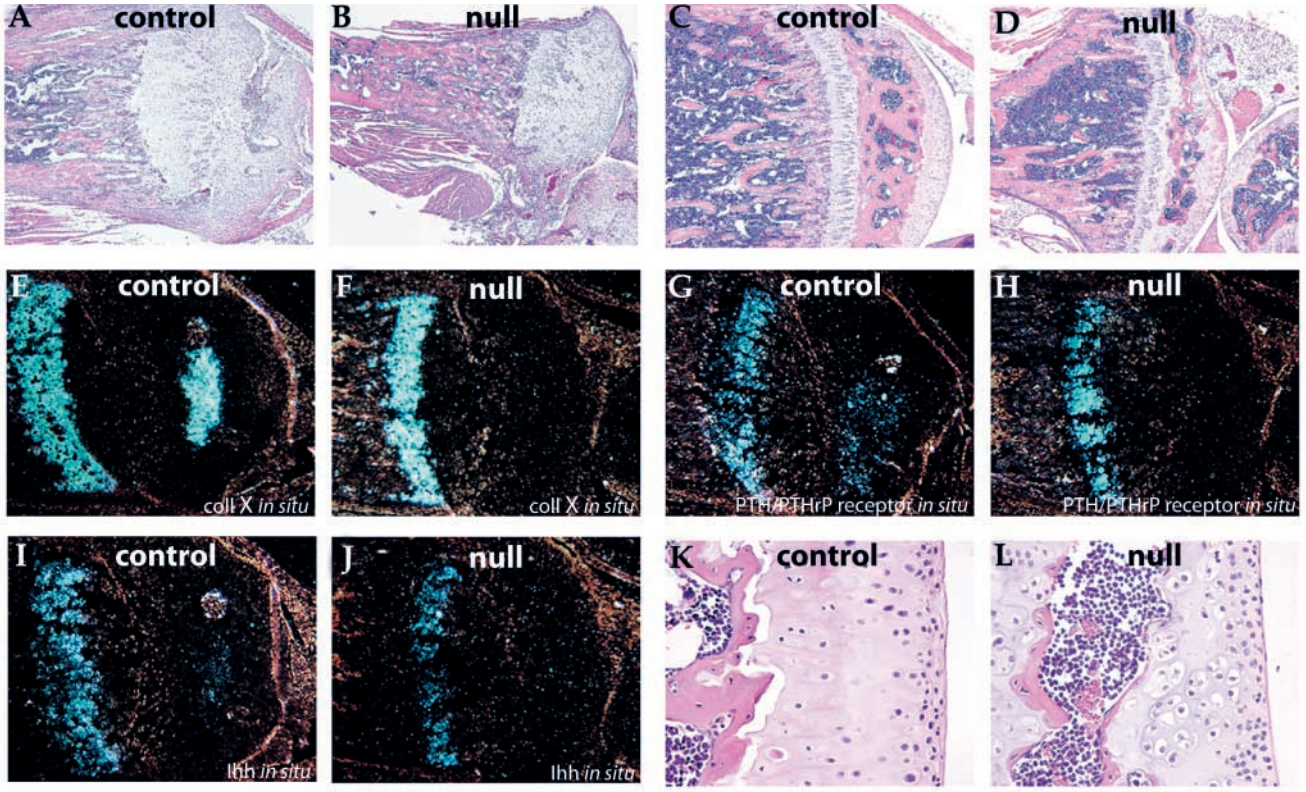

Fig. 4. Delay of the secondary ossification center in the *Vhlh* null growth plate. (A-D) Hematoxylin and Eosin staining of histological sections of control (A,C) and null (B,D) proximal tibia growth plates from 5-day- (A,B), and 1-month-old (C,D) mice. (E-J) In situ hybridization analysis of histological sections of tibia proximal growth plates from control (E,G,I) and *Vhlh* null (F,H,J) 5-day-old mice, with type X collagen (E,F), PTH/PTHrP receptor (G,H) and Indian Hedgehog (I,J) cRNA; darkfield images are shown. (K,L) Articular cartilage of control and *Vhlh* null animals, one month after birth, at higher magnification.

the proliferating and in the resting zones, compared with control specimens throughout fetal development (Fig. 5E).

Notably, deletion of *Hif1a* leads to decreased expression of $p57^{kip2}$ (CDKN1C – Mouse Genome Informatics) transcripts in growth plate chondrocytes (Schipani et al., 2001). $p57^{kip2}$, which is a CDK inhibitor, is involved in the exit of epiphyseal chondrocytes from the cell cycle (Nagahama et al., 2001; Yan et al., 1997). Having shown that both proliferation rate and cell numbers were significantly reduced in the absence of pVHL, we next determined $p57^{kip2}$ mRNA levels in the *Vhlh* null growth plate. In situ hybridization analysis of histological sections from newborn hindlimb showed that $p57^{kip2}$ mRNA expression was indeed upregulated in the null growth plate, especially in the resting and proliferating layers compared with in control specimens (Fig.5C,D). This finding suggests that $p57^{kip2}$ might be involved in the modulation of chondrocyte proliferation by pVHL.

Expression of HIF1α-dependent genes is upregulated in the *Vhlh* null growth plate

We next investigated whether the transcription factor HIF1α is stabilized and accumulates in the absence pVHL. In the *Vhlh* null growth plate, the intensity and number of chondrocytes that stained positive for HIF1α protein were clearly increased, demonstrating that deletion of *Vhlh* indeed leads to an accumulation of HIF1α (Fig. 6A,B). Western blot analysis of protein lysates of primary chondrocytes grown in normoxic conditions confirmed a dramatic accumulation of HIF1α hydroxylated at Pro564 in cells lacking *Vhlh*, when compared with the control (Fig. 7A).

Next we analyzed the transcriptional activity of HIF1α in control and mutant growth plates. HIF1α increases the expression of enzymes of the glycolytic pathway, and of angiogenetic factors such as VEGF (Firth et al., 1995; Ryan et al., 1998; Seagroves et al., 2001; Semenza, 2000). Consistent with these findings, hypoxic growth plate chondrocytes lacking functional HIF1α display downregulation of PGK and VEGF mRNA expression, both in vitro and in vivo (Pfander et al., 2003; Schipani et al., 2001). We thus hypothesized that lack of pVHL would lead to an increased synthesis of both glycolytic enzymes and VEGF through increased accumulation of HIF1α.

In situ hybridization analysis of newborn wild-type epiphysis revealed that PGK transcripts were expressed in the center of both the proliferative and prehypertrophic, with a typical outside-inside pattern that resembled the pattern of oxygenation of the growth plate (Fig. 6C) (Schipani et al., 2001). By contrast, in the newborn mutant growth plate, the outside-inside pattern of expression of PGK mRNA was perturbed; PGK mRNA was detectable throughout the whole epiphysis, including in a few scattered hypertrophic chondrocytes (Fig. 6D). Furthermore, resting chondrocytes located in close proximity to the articular surface showed an increased expression of PGK mRNA (Fig. 6D).

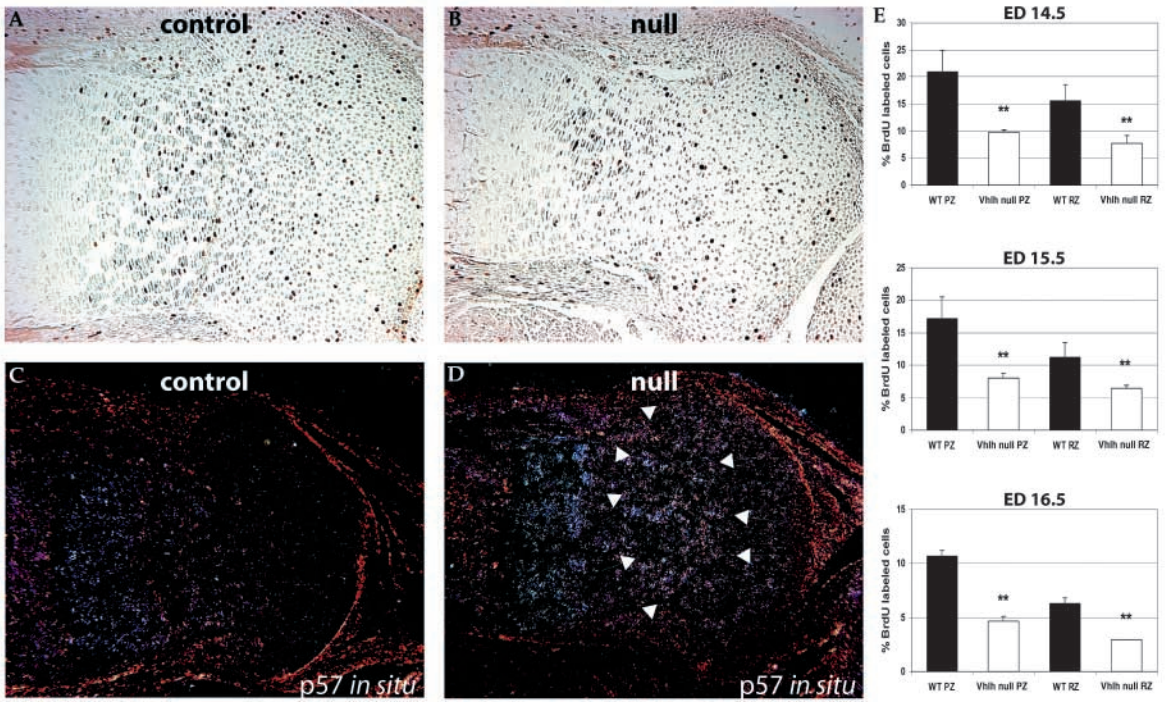

Fig. 5. Evidence for a decreased cell proliferation rate within the *Vhlh* null growth plate. (A,B) Detection of BrdU-labeled chondrocytes in histological sections of E16.5 control (A) and *Vhlh* null (B) proximal tibia growth plates. (C,D) In situ hybridization analysis with $p57^{kip2}$ cRNA on histological sections of newborn control (C) and *Vhlh* null (D) growth plates; darkfield images are shown. (E) Percentage of BrdU-labeled cells; bars represent mean percentages (±s.d.) of BrdU-labeled chondrocytes in *Vhlh* null and wild-type (WT) growth plates at E14.5, E15.5 and E16.5, separated into proliferating zone (PZ) and resting zone (RZ). Statistical differences in each zone were identified using the unpaired *t*-test. $^{**}P<0.01$.

We then analyzed VEGF mRNA expression by in situ hybridization. Our in situ hybridization analysis showed that in the newborn wild-type growth plate, VEGF mRNA was present not only in hypertrophic chondrocytes, but also, although to a lesser extent, in the center of the proliferative zone (Fig. 6E). In the newborn mutant growth plate, similar to PGK transcripts, VEGF mRNA was detectable in all layers. In addition, VEGF mRNA expression appeared to be granular and was upregulated in chondrocytes located next to the articular cap (Fig. 6F). Similar mRNA expression patterns of VEGF and PGK were observed in postnatal life (data not shown).

Consistent with these findings, measurements of soluble VEGF in the supernatant of primary chondrocytes grown in normoxic conditions, and real-time PCR of total RNA isolated from the same cells confirmed a dramatic accumulation of both VEGF protein and mRNA in cells lacking *Vhlh* when compared with controls (Fig. 7B,C). Furthermore, PGK mRNA levels were elevated in *Vhlh* null chondrocytes when compared with controls, even if to a lesser degree than VEGF mRNA (Fig. 7C).

Taken together, these findings strongly support the hypothesis that lack of pVHL in chondrocytes leads to an accumulation of HIF1α protein and to an increased expression of its target genes.

Growth plates lacking both *Vhlh* and *Hif1a* display the *Hif1a null* phenotype

In the final set of our experiments, we investigated whether the unique phenotype in mice lacking functional pVHL resulted from accumulation of the transcription factor HIF1α and through activation of HIF1α target genes. To address this question, we created double mutant mice lacking both the transcription factor HIF1α and pVHL in cartilage (*Vhlh*/*Hif1a* null). These mice died within the first hours after birth, like the mice that lack only HIF1α in cartilage (*Hif1a* null). The limbs of *Vhl*/*Hif1a* null newborns were essentially identical to the limbs isolated from *Hif1a* null newborns (data not shown). Both types of mutant limbs were much shorter and were deformed compared with wild-type controls (Fig. 8H-J). Furthermore, as in the *Hif1a* null growth plate, the center of the *Vhl*/*Hif1a null* growth plate was remarkably hypocellular as result of massive cell death, which generated a spatially localized defect extending from the joint space to the primary spongiosa (Fig. 8H-J). Lastly, similar to the *Hif1a* null growth plate, the proliferation index of the viable chondrocytes surrounding the central area of cell death was significantly increased in the *Vhl*/*Hif1a* null growth plate in comparison with controls (Fig. 8D-G). Both mutant phenotypes were extremely severe at birth, and were already clearly evident at E.14.5 (Fig. 8A-C).

Taken together, the deletion of both *Hif1a* and *Vhlh* apparently neutralized the phenotypic alterations seen in the *Vhlh* null growth plate, and generated a growth plate phenotype that was virtually indistinguishable from the phenotype caused by deletion of *Hif1a* alone.

Discussion

This is the first study reporting the novel finding that pVHL

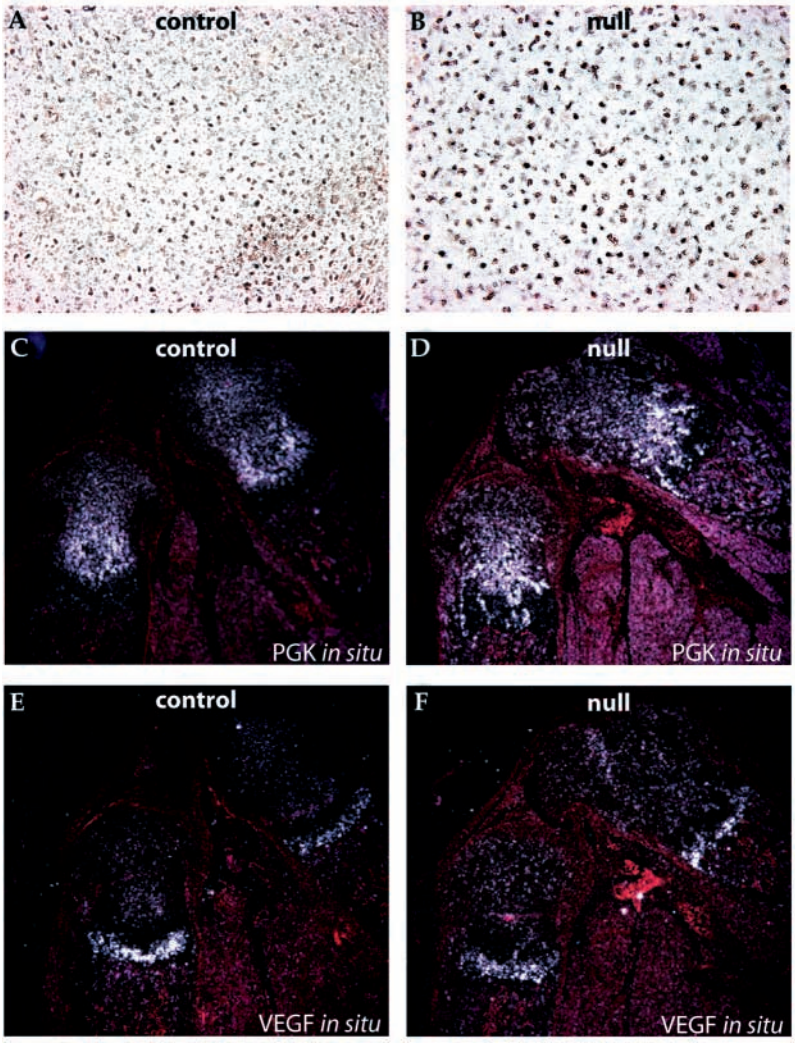

Fig. 6. (A,B) Evidence for stabilization of HIF1α in the *Vhlh* null growth plate. Immunohistochemical detection of HIF1α in cryosections of newborn control (A) and *Vhlh* null (B) proximal tibia growth plates.
(C-F) Evidence for an increased HIF1α activity in *Vhlh* null growth plates. In situ hybridization analyses of histological sections of proximal tibia growth plates from control (C,E) and *Vhlh* null (D,F) newborns, with PGK (C,D) and VEGF (E,F) cRNA; darkfield images are shown.

has a crucial role in endochondral bone development. Mice lacking *Vhlh* in the cartilaginous elements as result of the Cre-loxP strategy displayed a unique cartilage phenotype. This phenotype was characterized by hypocellularity, a dramatic decrease of cell proliferation, an augmented amount of matrix, and an increased cell size in the resting and proliferating zones. After birth, *Vhlh* null mice showed a delayed appearance of the secondary ossification center and severe dwarfism.

The VHL tumor suppressor protein is required to regulate HIF1α, and inactivation of VHL has been linked to the development of a variety of highly angiogenic tumors, including hemangioblastoma of the retina and central nervous system, clear cell carcinoma of the kidney, and pheochromocytoma (Maher and Kaelin, 1997). Loss of pVHL function leads to an increased stability of HIF1α and overexpression of proteins encoded by HIF1α target genes, such as VEGF and PGK (Gnarra et al., 1996; Cramer et al., 2003). However, although HIF1α is the best characterized pathway that is affected by pVHL loss of function, pVHL has been reported to have a range of effects within the cells that are not clearly related to HIF-1α activation. Identification of HIF1α-dependent and -independent effects of pVHL action is thus an open field of intense investigation.

In this study, we report the novel and intriguing finding that the tumor suppressor protein pVHL is necessary for normal chondrocyte proliferation in vivo. *Vhlh* null growth plates show a dramatic decrease in chondrocyte proliferation at different stages of fetal development, as determined by BrdU labeling. The role of pVHL in regulating cell proliferation is not fully elucidated (Kondo and Kaelin, 2001). Our data differ from previous studies that have shown that lack of functional pVHL impairs cell cycle exit under certain experimental conditions (Bindra et al., 2002; de Paulsen et al., 2001; Pause et al., 1998). However, our findings are consistent with the recent report that lack of pVHL inhibits cell proliferation in a teratocarcinoma model (Mack et al., 2003). It is highly possible that pVHL actions on the cell cycle may indeed vary in different cell types. This hypothesis would be in agreement with the observation that, despite the ubiquitous pattern of *VHL* expression, only a restricted subset of tumors is observed in patients suffering from the von Hippel Lindau syndrome (Kaelin and Maher, 1998).

Conditional inactivation of HIF1α in chondrocytes leads to the increased proliferation rate of viable chondrocytes at the periphery of the growth plate, while central cells undergo massive cell death (Schipani et al., 2001). In addition, a concomitant decrease in the mRNA expression of the CDK-inhibitor $p57^{kip2}$ is also detectable in murine growth plates lacking HIF1α. $p57^{kip2}$ is apparently required for exit of epihyseal chondrocytes from the cell cycle (Nagahama et al., 2001; Yan et al., 1997). Upregulation of $p57^{kip2}$ has been identified by microarray analysis in renal epithelial cells exposed to hypoxia (Leonard et al., 2003). Consistent with these findings, *Vhlh* deficient growth plates displayed a mild but consistent increase in $p57^{kip2}$ mRNA expression, as shown by in situ hybridization analysis. This increased expression of $p57^{kip2}$ transcripts may be partly responsible for the hypocellularity and the decreased proliferation rate observed in *Vhlh* null growth plates. However, chondrocyte proliferation is regulated at multiple levels by numerous factors, such as FGFs, BMPs, PTHrP, IHH, cell-cell and cell-matrix adhesion, and biomechanical signals (Shum et al., 2002). It is therefore likely that regulation of chondrocyte proliferation by pVHL does not involve only modulation of $p57^{kip2}$ expression. It will be now interesting to study whether and how the pVHL/HIF1α system interacts and cooperates with all the different pathways that regulate cell proliferation in the developing growth plate.

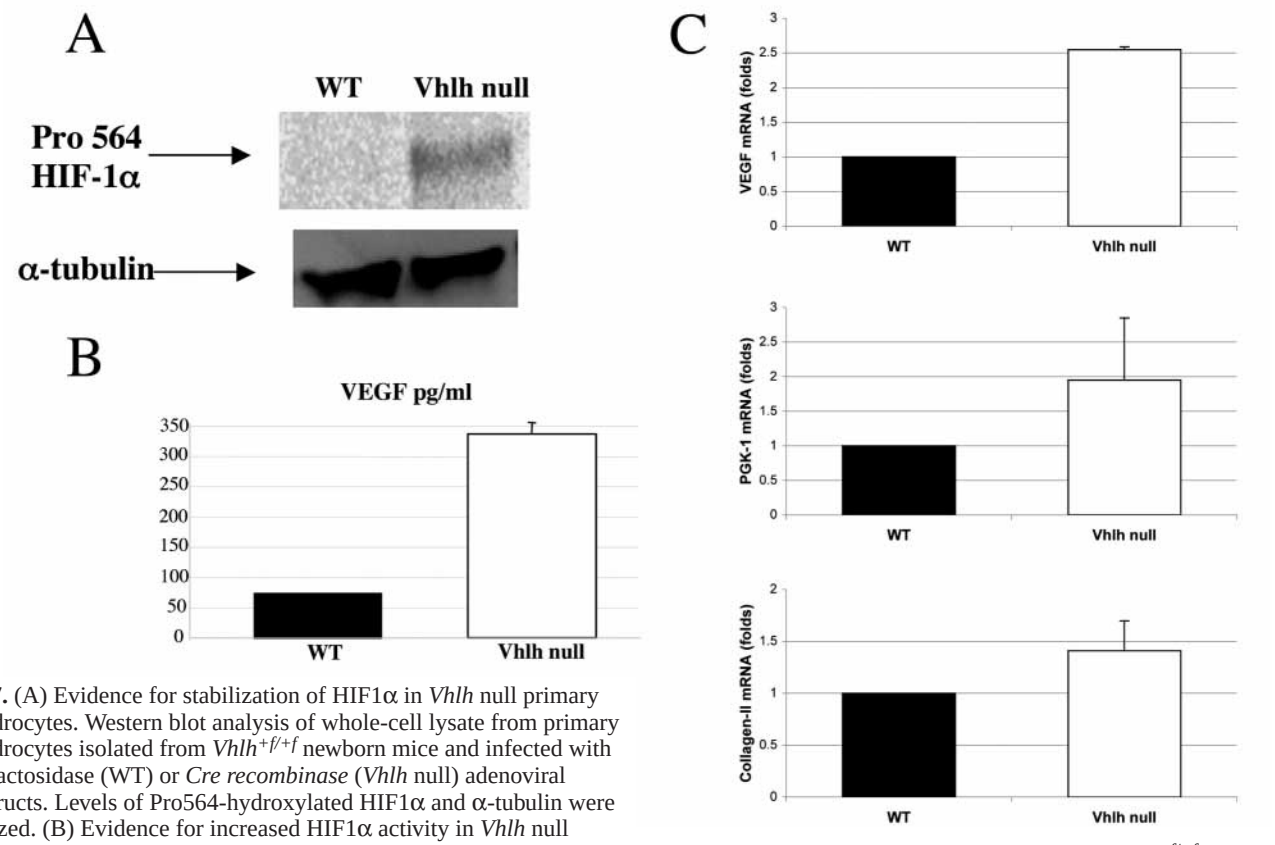

Fig. 7. (A) Evidence for stabilization of HIF1α in *Vhlh* null primary chondrocytes. Western blot analysis of whole-cell lysate from primary chondrocytes isolated from $Vhlh^{+f/+f}$ newborn mice and infected with β-galactosidase (WT) or *Cre recombinase* (*Vhlh* null) adenoviral constructs. Levels of Pro564-hydroxylated HIF1α and α-tubulin were analyzed. (B) Evidence for increased HIF1α activity in *Vhlh* null primary chondrocytes. Elisa measurements of soluble VEGF in the supernatant from primary chondrocytes isolated from $Vhlh^{+f/+f}$ newborn mice and infected with β-galactosidase (WT) or *Cre recombinase* (*Vhlh* null) adenoviral constructs. (C) VEGF, PGK and type II collagen mRNA expression measured by real-time PCR of total RNA isolated from primary chondrocytes isolated from $Vhlh^{+f/+f}$ newborn mice and infected with β-galactosidase (WT) or *Cre recombinase* (*Vhlh* null) adenoviral constructs.

The resting zone of the *Vhlh* null growth plate was mainly occupied by enlarged chondrocytes with a high cytoplasm to nucleus ratio, with similarities to pre-hypertrophic or hypertrophic chondrocytes. However, no evidence of ectopic hypertrophic differentiation could be found by analyses of type X collagen, PPR or IHH mRNA expression. It is possible that the decreased mitotic activity is the pathogenic event that leads to both the hypocellularity and the appearance of atypical cells observed in the *Vhlh* null growth plate. However, to our knowledge this unique cartilage phenotype characterized by increased cell size and reduced proliferation has not been described in other knockout models in which chondrocyte proliferation has been reported to be severely impaired, such as the *Ihh* knockout (St-Jacques et al., 1999). Therefore, the increased cell size observed in the *Vhlh* null growth plate probably is not the necessary or direct consequence of the decreased cell proliferation per se. It is possible that lack of pVHL in chondrocytes uncouples cell size and cell proliferation by regulating a still unknown molecular mechanism. Alternatively, pVHL may regulate chondrocyte size and shape through pathways that are distinct from those involved in chondrocyte proliferation. In this regard, it has been recently reported that pVHL is a microtubule-associated protein that can protect microtubules from depolymerization in vivo (Hergovich et al., 2003), and this function appears to be independent of its ability to engage in E3 ligase complex formation.

It is well known that chondrocyte proliferation and differentiation require their attachment to the matrix (Terpstra et al., 2003). In our in vivo model, the lack of *Vhlh* in cartilage leads not only to decreased proliferation, but also to increased matrix between cells, as shown by histological analysis. Hypoxia increases type II collagen accumulation in vitro in a HIF1α-dependent manner (Kurz et al., 2001; Pfander et al., 2003). Furthermore, recently it has been shown that hypoxia increases a group of procollagen hydroxylases that are indispensable for collagen fiber formation through stabilization of the transcription factor HIF1α (Hofbauer et al., 2003). It is thus highly possible that the increased matrix deposition observed in *Vhlh* null growth plates might result from accumulation of HIF1α in the mutant chondrocytes, leading to both an increased expression of type II collagen mRNA and to an enhanced synthesis of procollagen hydroxylases that are critically required for collagen triple helix formation. However, at this stage of investigation, a HIF1α-independent role of pVHL in regulating matrix accumulation cannot be excluded. Notably, it has been shown that pVHL can bind directly to fibronectin, a very important matrix protein (Ohh et al., 1998). A specific role of pVHL in extracellular matrix formation is also suggested by the finding that pVHL regulates metalloproteinases production and activity (Koochekpour et al., 1999). Further investigation will be needed in order to establish the role of pVHL in the regulation of cartilage matrix and, more generally, in chondrogenesis. It will be then crucial

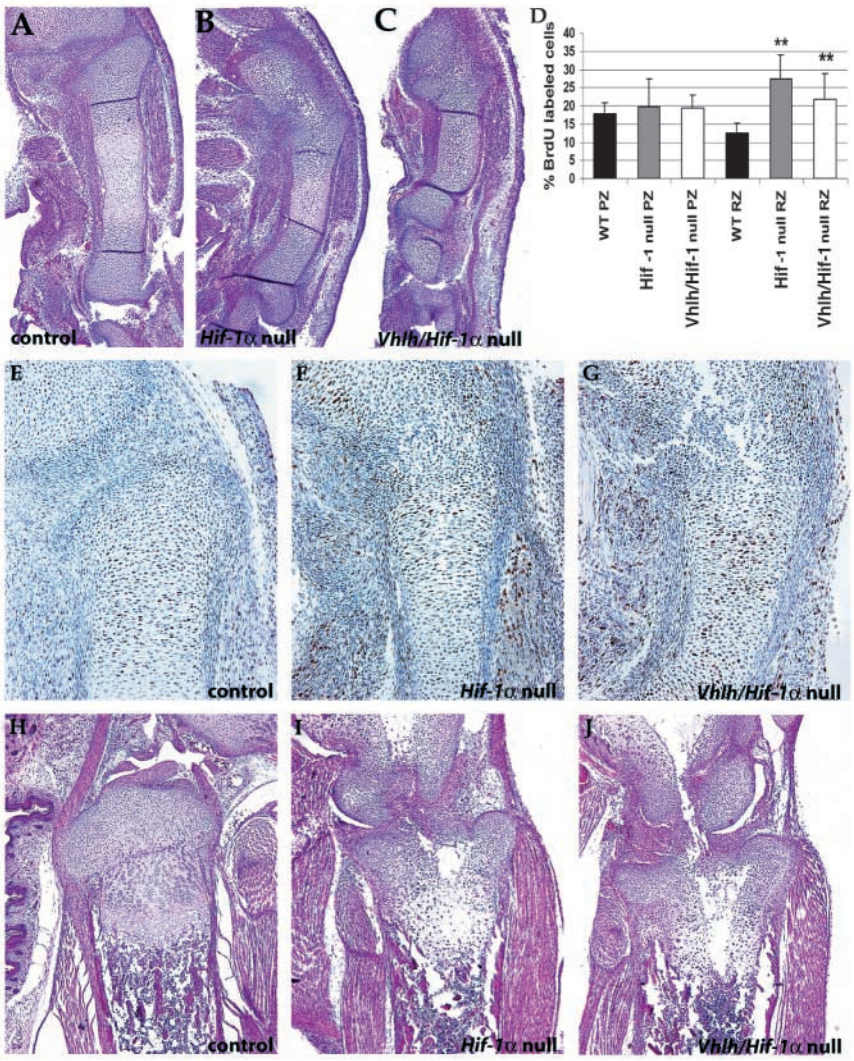

Fig. 8. Analysis of growth plates from control, *Hif1a* null and *Hif1a/Vhlh* null mice. (A-C,H-J) Hematoxylin and Eosin staining of histological sections of control (A,H), *Hif1a* null (B,I) and *Hif1a/Vhlh* null mice (C,J), at E14.5 (A-C) and birth (H-J). (E-G) Evidence for increased cell proliferation rates in *Hif1a* null and *Hif1a/Vhlh*null chondrocytes. Detection of BrdU-labeled chondrocytes in histological sections of E14.5 control (E), *Hif1a* null (F) and *Hif1a/Vhlh* null (G) proximal tibia growth plates. (D) Percentage of BrdU-labeled cells; bars represent mean percentages (±s.d.) of BrdU-labeled chondrocytes in *Hif1a* null, *Hif1a/Vhlh* null and wild-type (WT) growth plates at E14.5, separated into proliferating zone (PZ) and resting zone (RZ). Statistical differences in each zone were identified using the unpaired *t*-test. $^{**}P<0.01$.

to identify whether this role is indeed dependent on HIF1α transcriptional activity.

VEGF is one of the best indicators of HIF1α transcriptional activity; consistent with this finding is the observation that tumors caused by *Vhlh* inactivation are highly angiogenic and accumulate HIF1α. Blood vessel invasion of the epiphysis, a crucial step in endochondral bone development, has been shown to be regulated in part by VEGF activity (Gerber et al., 1999; Horner et al., 1999; Maes et al., 2002; Zelzer et al., 2002; Ortega et al., 2003; Haigh et al., 2000; Carlevaro et al., 2000). Several groups have reported that VEGF mRNA expression is mainly restricted to the hypertrophic zone (Gerber et al., 1999; Horner et al., 1999). Our data show that VEGF mRNA is also produced by proliferating chondrocytes, suggesting a broader role of VEGF in chondrocyte biology. In agreement with this hypothesis, more recent reports indicate that VEGF is an important modulator of chondrocyte differentiation, and it is also a critical survival factor for chondrocytes (Zelzer et al., 2002; Maes et al., 2004; Zelzer et al., 2004).

VEGF expression in chondrocytes is in part regulated by HIF1α-dependent mechanisms (Schipani et al., 2001; Pfander et al., 2003). Consistent with this notion, in the present study we detected a change in the expression pattern of VEGF mRNA in the resting and proliferating zones of *Vhlh* null growth plates, probably caused by the stabilization of HIF1α. Interestingly, despite increased levels of VEGF expression in *Vhlh* null resting-zone chondrocytes, no ectopic blood vessel invasion was observed at this site. Furthermore, a delay of angiogenesis at the secondary ossification site was clearly noticeable in the *Vhlh* null specimens. Taken together, these findings suggest that several other factors may be involved in regulating angiogenesis during endochondral bone development (Ivkovic et al., 2003), particularly at the secondary ossification center.

In our final experiment we investigated whether the morphological alterations, the decreased mitotic activity and the increased deposition of extracellular matrix molecules within the *Vhlh* null growth plate might result from the accumulation of HIF1α and the de-regulation of HIF1α target genes. To address this crucial question we used conditional inactivation of both *Vhlh* and *Hif1a* in all cartilaginous elements. The growth plate phenotype of *Hif1a*/*Vhlh* null mice was virtually indistinguishable from that observed in *Hif1a* null animals. This result is consistent with the idea that the altered endochondral ossification process observed in *Vhlh* null mice could result, at least in part, from increased activity of HIF1α, and, consequently, from de-regulation of HIF1α target gene expression. Further investigations will be needed to establish whether HIF1α is the only target of pVHL activity in growth plate chondrocytes.

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