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The Gli2 transcriptional activator is a crucial effector for Ihh signaling in osteoblast development and cartilage vascularization

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Indian hedgehog (Ihh) critically regulates multiple aspects of endochondral bone development. Although it is generally believed that all Ihh functions are mediated by the Gli family of transcription activators and repressors, formal genetic proof for this notion has not been provided. Moreover, the extent to which different Gli proteins contribute to Ihh functions is not fully understood. Previous work has shown that de-repression of the Gli3 repressor is the predominant mode through which Ihh controls chondrocyte proliferation and maturation, but that osteoblast differentiation and hypertrophic cartilage vascularization require additional mechanisms. To test the involvement of Gli2 activation in these processes, we have generated a mouse strain that expresses a constitutive Gli2 activator in a Cre-dependent manner, and have attempted to rescue the *Ihh*-null mouse with the Gli2 activator, either alone or in combination with Gli3 removal. Here, we report that the Gli2 activator alone is sufficient to induce vascularization of the hypertrophic cartilage in the absence of Ihh but requires simultaneous removal of Gli3 to restore osteoblast differentiation. These results therefore provide direct genetic evidence that Gli2 and Gli3 collectively mediate all major aspects of Ihh function during endochondral skeletal development.

KEY WORDS: Ihh, Gli2, Gli3, Bone, Cartilage, Vascularization, Mouse

INTRODUCTION

Much of the mammalian skeleton is derived from cartilage anlagen through endochondral bone formation. In the limb, mesenchymal cells that originate from the lateral plate mesoderm condense to form cartilage anlagen comprising a core of chondrocytes surrounded by the perichondrium. Following the initial proliferation that drives cartilage elongation, chondrocytes at the mid-section of the anlage exit the cell cycle and undergo hypertrophy. Subsequent to chondrocyte hypertrophy, de novo osteoblast differentiation occurs within the perichondrium surrounding the hypertrophic region to form a nascent bone collar. Concomitantly, blood vessels invade the hypertrophic cartilage to initiate cartilage erosion, bone deposition and marrow formation within the cartilage template. Overall, both perichondrial osteoblast differentiation and cartilage vascularization are crucial for transforming the cartilage anlage into a mature long bone, and both events are tightly regulated by signals produced by chondrocytes.

A key signal emanating from the developing chondrocytes is Indian hedgehog (Ihh), one of the three mammalian Hedgehog (Hh) proteins. Ihh is primarily expressed by prehypertrophic (immediately before hypertrophy) and early hypertrophic chondrocytes, but signals to both proliferating chondrocytes and the overlying perichondrial cells (St-Jacques et al., 1999; Vortkamp et al., 1996). *Ihh*$^{-/-}$ mice exhibit profound defects in chondrocyte proliferation and hypertophy, hypertrophic cartilage vascularization and osteoblast differentiation (Long et al., 2001; St-Jacques et al., 1999). As with all Hh proteins, Ihh signals through the seven-pass transmembrane protein smoothened (Smo) in the recipient cell. Genetic manipulations of *Smo* in Ihh recipient cells revealed that Ihh directly controls chondrocyte proliferation and osteoblast differentiation (Long et al., 2004; Long et al., 2001), but functions through parathyroid hormone-related peptide (Pthrp; Pthlh – Mouse Genome Informatics) to regulate chondrocyte maturation (Karp et al., 2000; Long et al., 2001; St-Jacques et al., 1999). In addition, Ihh appears to stimulate vascularization of the hypertrophic cartilage independent of signaling in either chondrocytes or endothelial cells, as *Smo* deletion in chondrocytes does not overtly affect vascularization (Long et al., 2001), and *Smo*$^{-/-}$ cells contribute to the bone marrow endothelium in chimeric embryos developed from wild-type and *Smo*$^{-/-}$ embryonic stem (ES) cells (Long et al., 2004). Thus, Ihh controls different aspects of skeletal development through either direct or indirect mechanisms.

The transcriptional effectors responsible for the various functions of Ihh are not completely understood. In general, the Gli transcription factors (Gli1/2/3) are believed to collectively mediate all Hh signaling in mammals. Among them, Gli2 and Gli3 are the primary responders, whereas Gli1 may function to amplify the response but is dispensable during mouse embryogenesis (Bai et al., 2002; Ding et al., 1998; Matise et al., 1998; Mo et al., 1997; Motoyama et al., 1998; Park et al., 2000). Moreover, Gli2 appears to function predominantly as a transcriptional activator in vivo (Ding et al., 1998; Komori et al., 1997; Matise et al., 1998; Mill et al., 2003; Sasaki et al., 1999), whereas Gli3 functions primarily as a repressor (Li et al., 2004; Litingtung and Chiang, 2000; Litingtung et al., 2002), although an activator function for Gli3 and a repressor activity for Gli2 have also been reported in certain genetic contexts (Bai et al., 2004; Buttitta et al., 2003). In the context of Ihh signaling in skeletal development, we and others have revealed Gli3 as a primary repressor, and that its genetic removal bypasses the requirement for Ihh in proper chondrocyte proliferation and hypertrophy (Hilton et al., 2005; Koziel et al., 2005). However, Gli3 removal fails to restore osteoblast differentiation and hypertrophic cartilage vascularization in the *Ihh*-null background, indicating that these processes require mechanisms independent of Gli3 de-repression. Interestingly, *Gli2*-null embryos exhibit deficiencies in

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both bone formation and cartilage angiogenesis (Miao et al., 2004). These results prompted us to hypothesize that the Gli2 activator might mediate Ihh signaling by regulating osteoblast differentiation and hypertrophic cartilage vascularization.

In this study, we have investigated the role of Gli2 activation, either alone or in combination with Gli3 de-repression, in skeletal development. We conclude that mediation of the full spectrum of Ihh function requires both Gli2 activation and Gli3 de-repression.

MATERIALS AND METHODS

DNA constructs

To generate the *ROSA26* targeting construct, the cDNA for *ΔNGli2* (starting with methionine followed by amino acids 280-1544 of the mouse Gli2) (Mill et al., 2003; Sasaki et al., 1999) was first cloned into *pCIG* (Megason and McMahon, 2002) between the *Cla*I and *Eco*RV sites to produce *pCIG-ΔNGli2*. *pCIG-ΔNGli2* was then digested with *Cla*I and *Not*I to release the fragment containing *ΔNGli2* cDNA and an ensuing *IRES2-GFP* sequence. The isolated fragment was then inserted into *pBigT* (Srinivas et al., 2001) at the *Cla*I and *Not*I sites to generate *pBigT-ΔNGli2*. Lastly, *pBigT-ΔNGli2* was digested with *Pac*I and *Asc*I, and the fragment containing *ΔNGli2* was cloned into *pROSA-PAS* (Soriano, 1999; Srinivas et al., 2001) to produce the final construct *pR26-ΔNGli2*.

Mouse strains

To generate the *R26-ΔNGli2* mouse strain, *pR26-ΔNGli2* was linearized with *Swa*I and electroporated into RW-4 ES cells (Murine Embryonic Stem Cell Core, Siteman Cancer Center, Washington University Medical School). The ES clones were screened for homologous recombination by Southern blot analyses using a 500 bp *Xho*I-*Bam*HI fragment derived from the *ROSA26* promoter (Kisseberth et al., 1999). Chimeric mice were generated by injecting ES cells into C57BL6 blastocysts (Tg/KO Micro-injection Core, Department of Pathology and Immunology, Washington University Medical School). PCR with ear biopsy samples were performed to determine germline transmission and for subsequent genotyping (Soriano, 1999). The $Ihh^{+/-}$, $Gli3^{Xt\text{-}J}$ and *Col2-Cre* (line 3) mouse strains are as previously described (Long et al., 2001; Maynard et al., 2002; St-Jacques et al., 1999).

Analyses of mouse embryos

Wholemount skeletal staining was performed with Alizarin Red and Alcian Blue based on McLeod (McLeod, 1980). To assess skeletal phenotypes on sections, embryonic limbs were harvested in PBS and fixed in 10% buffered formalin overnight at room temperature. The fixed limbs were then processed and embedded in paraffin before sectioning at 6 μm. Limbs from E18.5 embryos were decalcified in 14% EDTA/PBS (pH 7.4) for 48 hours after fixation and before processing.

Limb sections were subjected to morphological and molecular analyses. For histology, sections were stained with Hematoxylin and Eosin (H&E). To distinguish cartilage versus bone matrix, sections were stained with Alcian Blue and Picro-sirius Red (Junqueira et al., 1979). In situ hybridization was performed using ^{35}S-labeled riboprobes as previously described (Hilton et al., 2005; Hu et al., 2005; Long et al., 2004; Long et al., 2001). The chondrocyte proliferation rate in embryos was assessed by peritoneal injection of BrdU into pregnant females, followed by immuno-detection of BrdU incorporation in DNA in limb sections of the embryos (Zymed Laboratories). The labeling index was scored from three sections per cartilage element, and in three wild-type versus mutant littermate pairs unless otherwise indicated. Endothelial cells were detected by Pecam immunohistochemistry as follows. Sections were de-paraffinized, submersed in quenching solution (3% H_2O_2 in methanol) for 10 minutes, and then treated with trypsin for 10 minutes and a denaturing solution (both from the Zymed BrdU Detection Kit) for 25 minutes at room temperature. The sections were then blocked with 10 % sheep serum in PBS for 30 minutes at room temperature before being incubated with monoclonal rat anti-mouse Pecam1 (BD Pharmingen, San Diego, CA, USA) in 5% sheep serum in PBS overnight at 4°C, and then with biotinylated anti-rat IgG (BD Pharmingen) in 5% sheep serum in PBS for 1 hour at room temperature. The sections were finally incubated with peroxidase-conjugated streptavidin (DAKO) for 30 minutes, and then with DAB solution (Zymed BrdU Detection Kit) for color reaction.

RESULTS

Constitutive activation of Gli2 promotes chondrocyte maturation

To assess the role of the Gli2 transcriptional activator in the absence of Ihh, we took advantage of an N-terminally truncated form of Gli2 (ΔNGli2, deletion of amino acids 1-279) that was previously shown to be constitutively active independent of Hh ligands. Specifically, we generated a mouse strain (termed *R26-ΔNGli2*) by knocking the *ΔNGli2* cDNA into the ubiquitously active *ROSA26* locus so that ΔNGli2 can be expressed in a Cre-dependent manner (Fig. 1A). To test the functionality of the *R26-ΔNGli2* mouse strain, we generated mice of genotype *Col2-Cre;*$R26^{\Delta NGli2/+}$ (termed *C2ΔNGli2*, with one *ΔNGli2* and one wild-type allele at the *ROSA26* locus) by using a *Col2-Cre* transgenic line that expresses Cre in the precursors of both chondrocytes and osteoblasts. The *C2ΔNGli2* mice survived postnatally but were consistently smaller than their littermate controls. We have focused on the embryos in this study.

The *C2ΔNGli2* embryos exhibited slight dwarfism. This was evident from wholemount skeletal staining, which showed that all limb long bones were reduced by 10-15% from the normal length at E18.5 (Fig. 1B). At the histological level, the long bones of *C2ΔNGli2* animals contained shorter proliferative and hypertrophic zones (Fig. 1Ca,a′). Consistent with histology, in situ hybridization confirmed that *Colα1(X)* (*Col10a1* – Mouse Genome Informatics), a specific marker for hypertrophic chondrocytes, was detected in a smaller domain, and closer to the articular surface (Fig. 1Cb,b′). The mechanism for the shorter hypertrophic zone in *C2ΔNGli2* embryos is uncertain at present but it is possible that hypertrophic chondrocytes progressed to the terminal stage (followed by apoptosis and removal) at a faster rate. Overall, the shortening of the long bones in *C2ΔNGli2* embryos correlates with dysregulation of the growth region cartilage.

To determine whether the shortening of the proliferative zone was due to premature hypertrophy, we examined the long bones at E14.5 prior to vascularization and removal of the hypertrophic cartilage. Indeed, the *C2ΔNGli2* embryo exhibited a longer hypertrophic, but a shorter proliferative, zone than its wild-type littermate (Fig. 1Da,a′). Measurements of the two subdomains against the total length of the element revealed that these deviations were statistically significant (Fig. 1E). Consistent with histology, *Colα1(II)* (*Col2a1* – Mouse Genome Informatics), a marker for proliferating chondrocytes, was expressed in a smaller domain than normal (Fig. 1Db-b′), whereas *Colα1(X)*, a marker for hypertrophic chondrocytes, was detected in a wider region than normal (Fig. 1Dc,c′). Importantly, the premature onset of hypertrophy was not due to diminution of Pthrp, as in situ hybridization detected no decrease in *Pthrp* levels in *C2ΔNGli2* embryos compared with the wild-type littermates (Fig. 1Dd,d′). Moreover, the shortening of the proliferative region was not caused by changes in the proliferation rate, as BrdU labeling experiments indicated that the labeling index was similar between *C2ΔNGli2* and the control littermates (Fig. 1F). Thus, the reduction in cartilage growth in the *C2ΔNGli2* embryo was primarily due to premature onset of hypertrophy independent of Pthrp levels.

To confirm that Ihh signaling was indeed activated by *ΔNGli2*, we examined the expression of *Ptch1*, a direct transcriptional target of Hh signaling. At E14.5, Ptch1 is normally expressed in the proliferating chondrocytes in a graded fashion, with the highest level closest to the hypertrophic zone, but with no expression in the hypertrophic chondrocytes (Fig. 1De). In the *C2ΔNGli2* embryo,

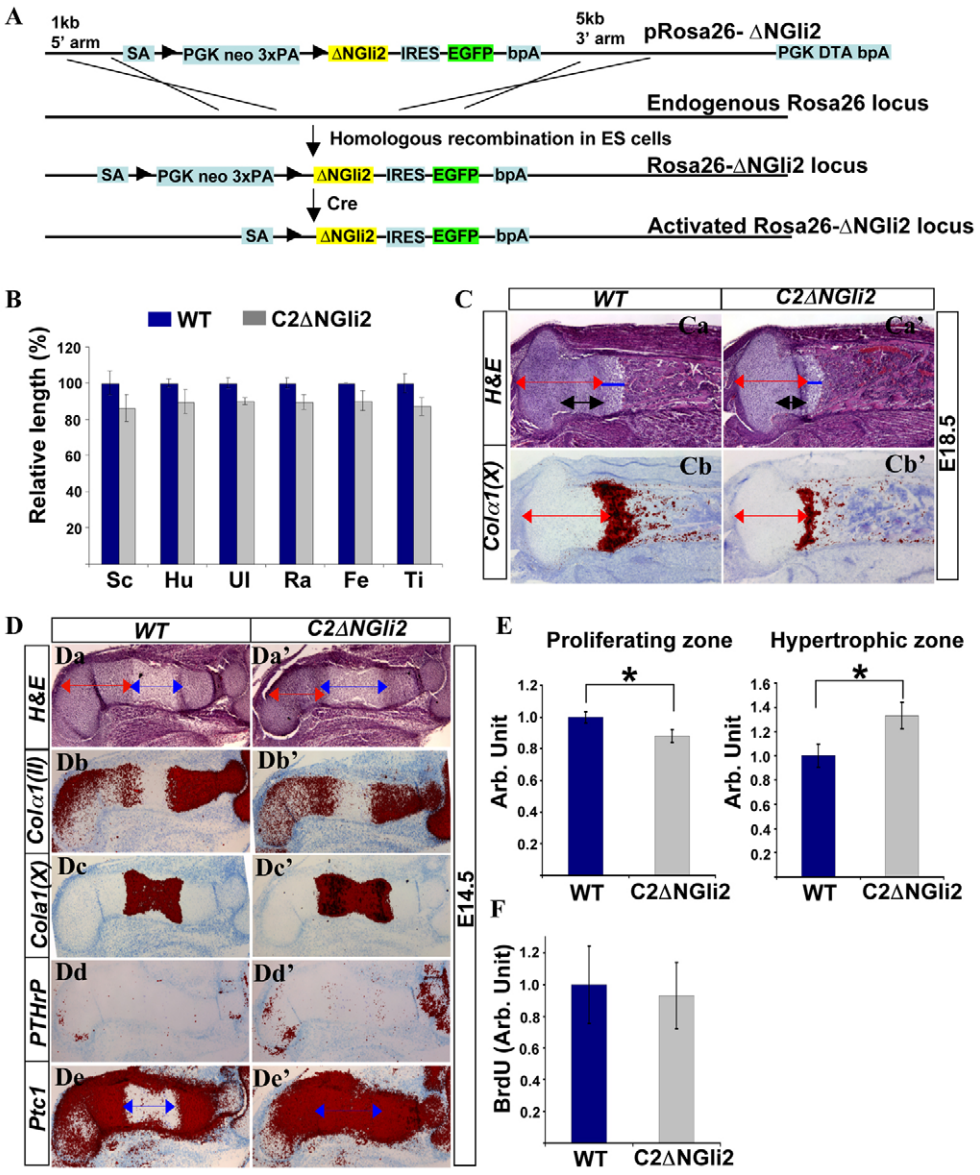

Fig. 1. Analyses of *Col2-Cre;R26$^{\Delta NGli2/+}$* (*C2ΔNGli2*) mouse embryos. (**A**) A schematic for the genetic strategy. (**B**) Relative bone lengths at E18.5. $P<0.05$, $n=5$. (**Ca-b'**) Analyses of longitudinal sections through the proximal half of the tibia at E18.5. (Ca,Ca') H&E staining. (Cb,Cb') In situ hybridization with ^{35}S-labeled riboprobes. Signal in red. Red double-headed arrows, proliferative zone; black double-headed arrows, columnar zone; blue line, hypertrophic zone. (**Da-e'**) Analyses of longitudinal sections through the humerus at E14.5. (Da,Da') H&E staining. (Db-De') In situ hybridization on adjacent sections. Red double-headed arrows, proliferative zone; blue double-headed arrows, hypertrophic zone. (**E**) Relative lengths of the proliferative and hypertrophic zones at E14.5 in WT versus mutant embryos. *, $P<0.05$, $n=3$. (**F**) Relative BrdU labeling index at E15.5. $P=0.73$, $n=3$. Fe, femur; Hu, humerus; Ra, radius; Sc, scapula; Ti, tibia; Ul, ulna.

however, strong *Ptch1* expression was detected throughout the cartilage including the hypertrophic zone (Fig. 1De'). Thus, ΔNGli2 markedly activated Hh signaling throughout the developing cartilage.

ΔNGli2 ameliorates defects in cartilage hypertrophy and vascularization in *Ihh*$^{-/-}$ embryos

To assess the contribution of Gli2 activator to Ihh functions in the endochondral skeleton, we generated mice of genotype *Col2-CRE;Ihh*$^{-/-}$*;R26*$^{\Delta NGLi2/+}$ (termed ΔNGli2-rescue). The animals died at birth, and were analyzed during embryogenesis. Histological analyses of E18.5 embryos revealed that the proliferative cartilage region was slightly longer in the ΔNGli2-rescue than in *Ihh*$^{-/-}$ embryos, but was still much shorter than in the wild type (Fig. 2Aa-c). Interestingly, the columnar organization of chondrocytes prior to hypertrophy, which was absent in the *Ihh*$^{-/-}$ embryos, was partially restored in the ΔNGli2-rescue embryos (Fig. 2Ba-c). Moreover, partial vascularization was observed in the hypertrophic zone of the ΔNGli2-rescue embryos, indicating a notable improvement over the *Ihh*$^{-/-}$ embryos that lacked any sign of vascularization (Fig. 2Ca-c).

To confirm the histological results, we performed molecular analyses. In keeping with the moderate lengthening of the proliferative zone, the *Colα1(X)*-positive cells were at a greater distance from the articular surface in the ΔNGli2-rescue embryos than in *Ihh*$^{-/-}$ littermates, although still much closer than normal (Fig. 2Da-c). We then probed the extent of cartilage vascularization by examining the expression of matrix metalloproteinase 9 (Mmp9), which marks the leading edge of blood vessel invasion, and Cd31 (Pecam1), which demarcates the endothelial cells. Consistent with improved vascularization in the ΔNGli2-rescue embryo, both Mmp9 and Pecam1 were detected at the core of the cartilage, but only peripherally in the *Ihh*$^{-/-}$ embryo (Fig. 2Ea-c,Fa-c,Ga-c). Overall, forced activation of Gli2 partially corrected the premature hypertrophy and the vascularization defect in the *Ihh*$^{-/-}$ background.

The increased length of proliferative cartilage led us to examine the expression of Pthrp and the proliferation status of the chondrocytes. Similar to at E18.5, at E15.5 the ΔNGli2-rescue embryos also exhibited a moderately longer proliferative zone than their *Ihh*$^{-/-}$ littermates (Fig. 3Aa-c). At this stage, Pthrp is normally expressed by peri-articular chondrocytes, but expression was absent

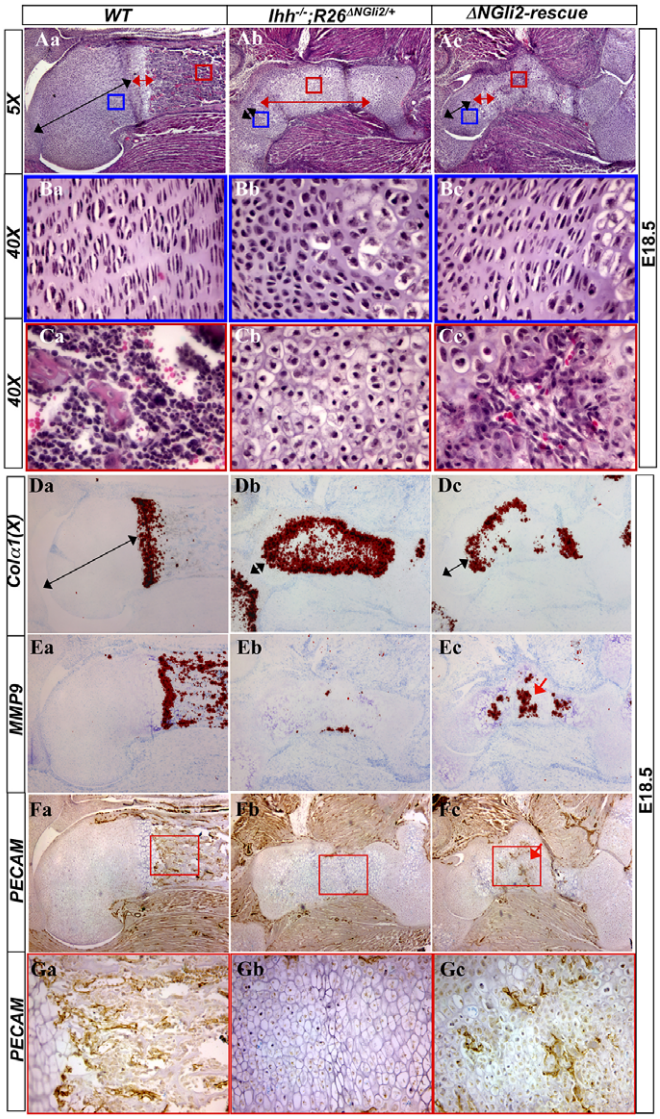

Fig. 2. Analyses of *Col2-Cre;Ihh*$^{-/-}$*;R26*$^{\Delta NGLi2/+}$ (ΔNGli2-rescue) embryos at E18.5. (**Aa-c**) H&E staining of longitudinal humerus sections. Proximal end to the left. (**Ba-c**,**Ca-c**) Higher magnification of color-corresponding boxed areas in Aa-c. (**Da-c**,**Ea-c**) In situ hybridization with ^{35}S-labeled riboprobes on longitudinal sections of the humerus. Signal in red. (**Fa-c**) Cd31 immunohistochemistry on longitudinal sections of the humerus. (**Ga-c**) Higher magnification of areas boxed in Fa-c. Signal in brown. Black double-headed arrows, proliferative zone; red double-headed arrows, hypertrophic zone; red arrows, signal detected within cartilage proper.

in *Ihh*$^{-/-}$ embryos (Fig. 3Ba-b). Interestingly, Pthrp was weakly, but reliably, detectable in ΔNGli2-rescue embryos (Fig. 3Bc), which also restored Ptch1 expression in the peri-articular chondrocytes (Fig. 3Cc). BrdU labeling experiments revealed that ΔNGli2 increased chondrocyte proliferation to 75% of the wild-type level from the 50% normally observed in *Ihh*$^{-/-}$ embryos (Fig. 3F). Thus, changes in both proliferation and Pthrp expression might have contributed to the moderate lengthening of the proliferative zone in ΔNGli2-rescue embryos.

The partial recovery of the columnar region in the ΔNGli2-rescue embryos prompted us to examine chondrocyte maturation at the molecular level. At E15.5, *Colα1(II)* and *Colα1(X)* are normally largely restricted to proliferative and hypertrophic regions, respectively, with the two domains only partly overlapping (Fig. 3Da,Ea). By contrast, in *Ihh*$^{-/-}$ embryos, many *Colα1(X)*-positive cells retained *Colα1(II)* expression, producing a largely overlapping pattern (Fig. 3Db,Eb). Remarkably, in the ΔNGli2-rescue embryos, the normal patterns of *Colα1(II)* and *Colα1(X)* expression were restored (Fig. 3Dc,Ec). Thus, ΔNGli2 normalized the progression of hypertrophy in the *Ihh*$^{-/-}$ background.

ΔNGli2 fails to rescue orthotopic bone collar formation in *Ihh*$^{-/-}$ mutants

We next examined the status of osteoblast differentiation in ΔNGli2-rescue embryos. At E18.5, H&E staining of these embryos revealed bone deposition at the diaphyseal region of the humerus but not in the perichondrium flanking the hypertrophic region where a bone collar normally forms (Fig. 4Aa-c). This observation was confirmed by Sirius Red-Alcian Blue staining, which detected bone matrix only at the diaphysis but not at the normal position adjacent to the early hypertrophic cartilage (Fig. 4Ba-Cc). The mechanism supporting bone formation at the diaphysis in the ΔNGli2-rescue embryo is currently unknown, but appears to correlate with vascularization in the region (see Discussion).

To investigate osteoblast differentiation at the molecular level, we performed in situ hybridization for several osteoblast markers. At E18.5, in the wild-type embryos, *Runx2*, alkaline phosphatase (AP; Akp1 – Mouse Genome Informatics), bone sialoprotein (BSP; Ibsp – Mouse Genome Informatics), osterix (*Osx*; *Sp7* – Mouse Genome Informatics), and osteocalcin (OC; Bglap1 – Mouse Genome Informatics) were expressed normally in the bone collar that spans the hypertrophic and the marrow region (Fig. 4Da-Ha); however, none of the markers was detected in the perichondrium of the *Ihh*$^{-/-}$ embryos even though several of the markers were expressed in the hypertrophic chondrocytes, as previously observed (Fig. 4Db-Hb). Consistent with the histological analysis, the ΔNGli2-rescue embryos expressed osteoblast markers only in the diaphyseal region but not in areas adjacent to the early hypertrophic cartilage (Fig. 4Dc-Hc). Importantly, at E14.5, when no cartilage vascularization was evident in the ΔNGli2-rescue embryos, the osteoblast markers were not detected anywhere in the perichondrium, including the diaphysis, despite their expression in the hypertrophic chondrocytes (Fig. 4Ic-Lc). Thus, ΔNGli2 failed to rescue orthotopic osteoblast differentiation in *Ihh*$^{-/-}$ embryos, even though bone formed at the diaphysis where vascularization occurred.

Gli2 activation and Gli3 removal together restore orthotopic bone collar formation in *Ihh*$^{-/-}$ embryos

The lack of orthotopic osteoblast differentiation in the ΔNGli2-rescue embryos prompted us to hypothesize that Ihh-dependent osteoblast differentiation could require both de-repression of Gli3 and activation of Gli2. To test this hypothesis, we generated *Col2-Cre;Ihh*$^{-/-}$*;Gli3*$^{-/-}$*;R26*$^{\Delta NGli2/+}$ embryos (termed double-rescue embryos). The double-rescue mice developed to term but died at birth, and were therefore analyzed during embryogenesis.

Morphological analyses revealed remarkable normalization of the skeleton in double-rescue embryos. Wholemount skeleton staining of E18.5 embryos showed that the overall size of double-rescue embryos was significantly increased over *Ihh*$^{-/-}$ embryos, although they were still smaller than the wild-type embryo (Fig. 5A-C). The limbs of double-rescue embryos were also notably longer than those in *Ihh*$^{-/-}$*;Gli3*$^{-/-}$ embryos (Fig. 5D). Importantly, bone collars were clearly visible in the limbs of double-rescue embryos. H&E staining of limb sections confirmed that the bone collar, like that in wild-type

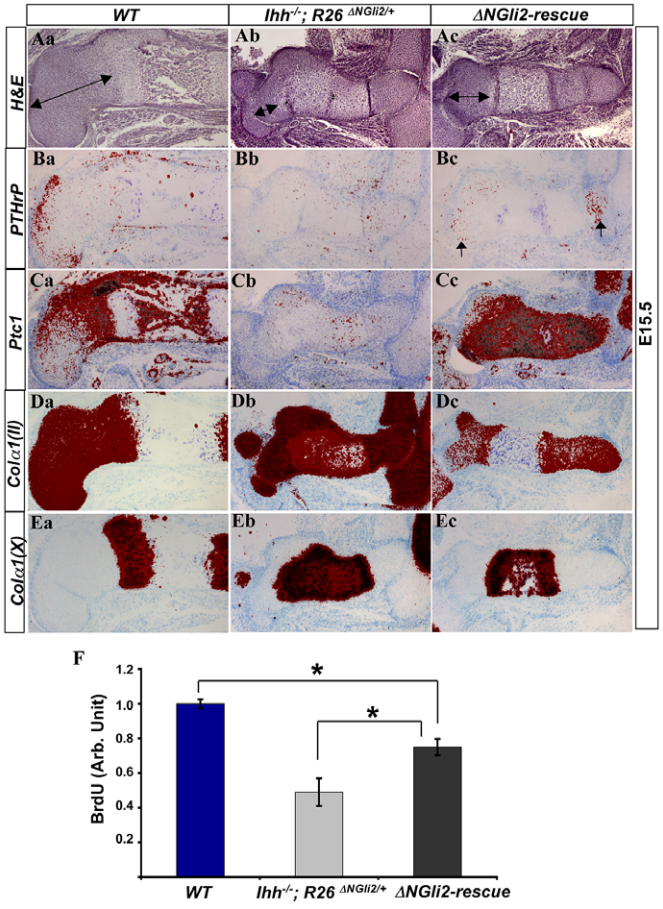

Fig. 3. Analyses of *Col2-Cre;Ihh*$^{-/-}$*;R26*$^{\Delta NGli2/+}$ (ΔNGli2-rescue) embryos at E14.5 and E15.5. (**Aa-c**) H&E staining of longitudinal sections through the humerus at E15.5. (**Ba-Ec**) In situ hybridization using ^{35}S-labeled riboprobes on adjacent sections. Proximal end to the left. Double-headed arrows, proliferative zone; arrows, faint expression of PTHrP. (**F**) Relative BrdU labeling index of chondrocytes in the distal half of the humerus at E14.5. *, $P<0.01$, $n=3$.

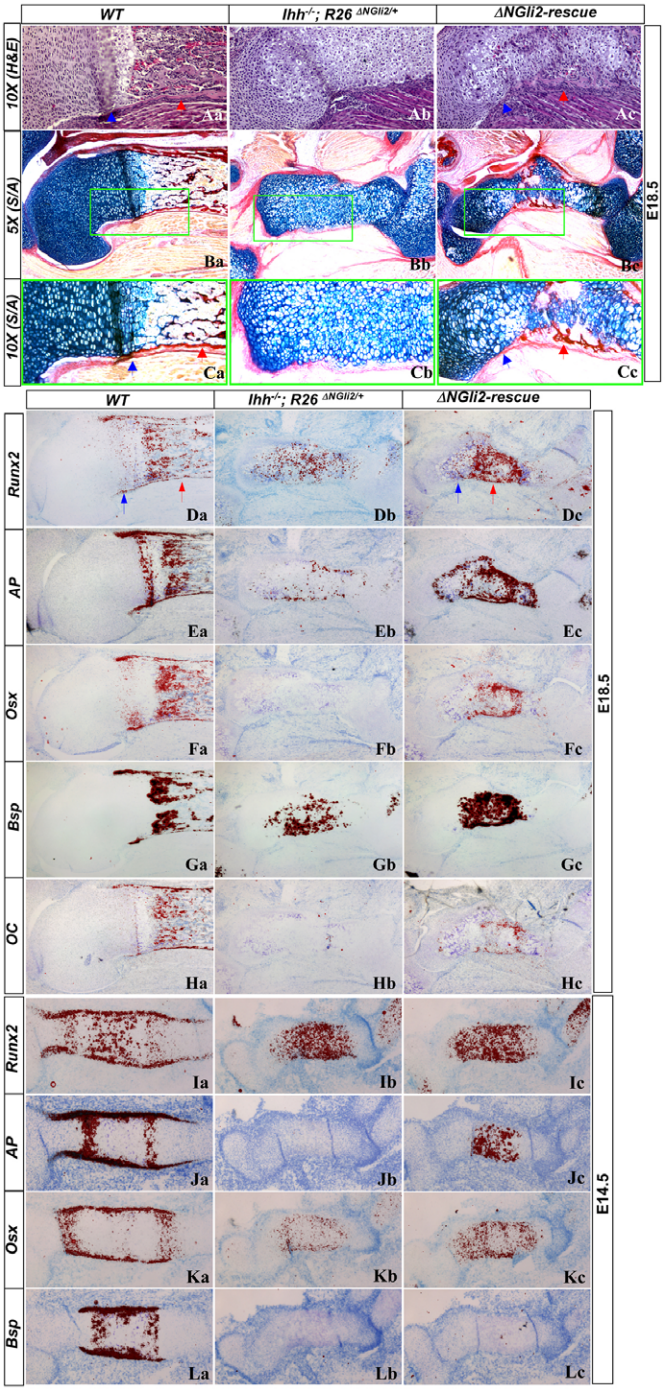

Fig. 4. Assessment of osteoblast development in *Col2-Cre;Ihh*$^{-/-}$*;R26*$^{\Delta NGli2/+}$ (ΔNGli2-rescue) embryos. (**Aa-c**) H&E staining. (**Ba-c**) Sirius Red and Alcian Blue staining. (**Ca-c**) Higher magnification of boxed areas in Ba-c. (**Da-Lc**) In situ hybridization using ^{35}S-labeled riboprobes for osteoblast markers. Signal in red. All sections longitudinal through the humerus, proximal end to the left. Blue arrows, perichondrium or bone collar flanking the early hypertrophic zone; red arrows, bone at diaphysis.

embryos, spanned the hypertrophic and the marrow region (Fig. 5E,Eb,F,Fb). This is in contrast to *Ihh*$^{-/-}$*;Gli3*$^{-/-}$ embryos, which did not form a bone collar in the perichondrium flanking the hypertrophic cartilage (Fig. 5G,Gb). In addition, the growth region cartilage was largely normal in double-rescue embryos, although it was longer than that in wild-type embryos (Fig. 5E,F), and the columnar zone contained areas of disorganization (Fig. 5Fd). Because the columnar zone was normal in the ΔNGli2-rescue (Fig. 2Bc) and *Ihh*$^{-/-}$*;Gli3*$^{-/-}$ (Fig. 5Gd) embryos, the abnormality seen in the double-rescue embryos might be due to ectopic gene activation by ΔNGli2 in the absence of Gli3. Finally, the marrow cavity and the hypertrophic chondrocytes in double-rescue embryos appeared to be normal (Fig. 5Ea,Ec,Fa,Fc). Conversely, *Ihh*$^{-/-}$*;Gli3*$^{-/-}$ embryos, as previously reported, did not develop a marrow cavity (Fig. 5Ga) even though hypertrophic chondrocytes looked normal (Fig. 5Gc). Thus, simultaneous removal of Gli3 and activation of Gli2 markedly improved skeletal morphology in the *Ihh*$^{-/-}$ background.

The morphological observations were corroborated by molecular analyses. Consistent with the longer growth plate at E18.5, *Colα1(X)* expression was detected both at a greater distance from the articular surface and within a wider domain in the double-rescue embryos than in wild-type embryos (Fig. 6Aa,b). The greater *Colα1(X)*-negative domain was indicative of a delay in the onset of hypertrophy. This delay was most evident at E15, when the hypertrophic cartilage that normally separates into two subdomains

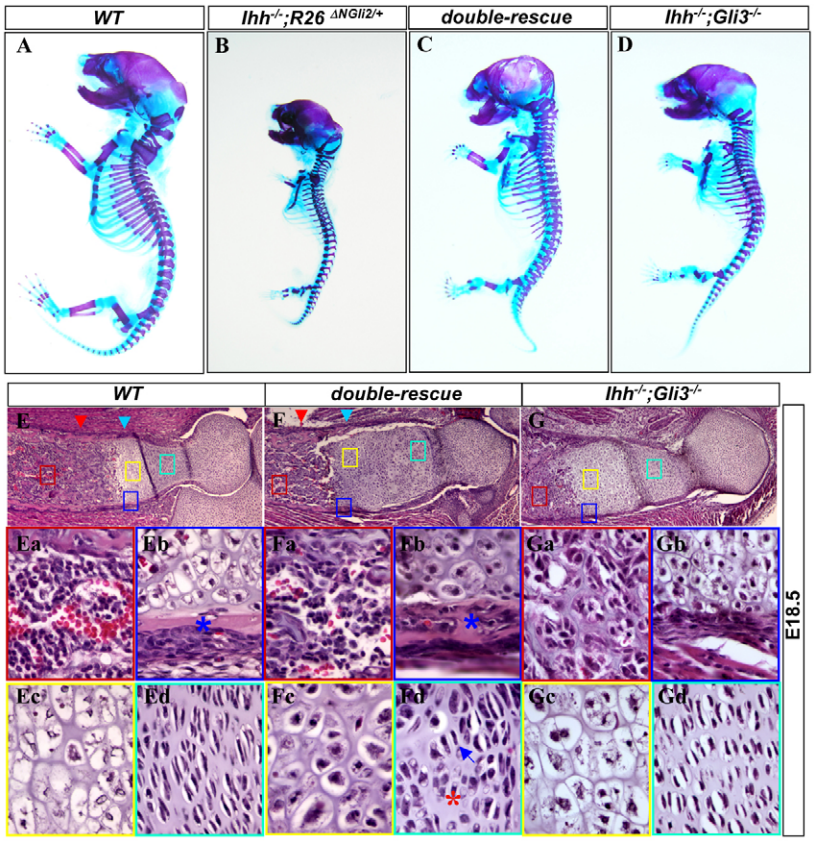

Fig. 5. Morphological analyses of *Col2-Cre;Ihh$^{-/-}$;Gli3$^{-/-}$;R26$^{\Delta NGli2/+}$* (double-rescue) and *Ihh$^{-/-}$;Gli3$^{-/-}$* embryos at E18.5. (**A-D**) Wholemount skeletal staining. (**E-G**) H&E staining of longitudinal sections through the humerus. Distal end to the right. Blue arrowheads, bone collar flanking the hypertrophic zone; red arrowheads, bone at diaphysis. (**Ea-Gd**) Higher magnification of areas in boxes with corresponding colors in E-G. Blue asterisks in Eb and Fb denote bone collar; red asterisk in Fd denotes a disorganized area in the columnar region; blue arrow in Fd points to a flat chondrocyte.

remained contiguous in the double-rescue embryos (Fig. 6Ba-Cb). Moreover, the delay in hypertrophy was probably due to the marked overproduction of Pthrp in the double-rescue embryos (Fig. 6Da,b). Conversely, the proliferation rate of chondrocytes was indistinguishable between the double-rescue and wild-type embryos (Fig. 6E). Overall, chondrocyte hypertrophy progressed in an orderly fashion in double-rescue embryos but exhibited a delay over the normal rate owing to Pthrp overproduction.

To confirm that bone formation was restored in the double-rescue embryo, we conducted further analyses. Sirius Red-Alcian Blue staining of limb sections confirmed a bone collar (stained dark red) surrounding the hypertrophic cartilage and the marrow cavity in both wild-type and double-rescue embryos at E18.5 (Fig. 7Aa,b). As previously reported, such a bone collar was not present in *Ihh$^{-/-}$;Gli3$^{-/-}$* embryos (Fig. 7Ac). In situ hybridization revealed that although *Runx2* expression in the perichondrium was similar among the three genotypes (Fig. 7Ba-c), *Osx* expression in the perichondrium flanking the hypertrophic cartilage was only detected in the wild-type and double-rescue embryos and not in the *Ihh$^{-/-}$;Gli3$^{-/-}$* embryos (Fig. 7Ca-c). Likewise, at E15, *Osx* and *Bsp* were detected in the expected domains in the perichondrium of the double-rescue embryo, but not in the *Ihh$^{-/-}$;Gli3$^{-/-}$* embryo, even though *Runx2* and AP were expressed in both embryos (Fig. 7Da-Gf). The shorter domains for the osteoblast markers in the double-rescue and *Ihh$^{-/-}$;Gli3$^{-/-}$* embryos corresponded to the delay in chondrocyte hypertrophy in these embryos compared with the wild type. Thus, the combination of Gli3 removal and Gli2 activation is sufficient to restore orthotopic bone collar formation in the absence of Ihh.

In summary, the present study, together with our previous work (Hilton et al., 2005), has revealed that the Gli2 activator and the Gli3 repressor execute distinct Ihh functions during skeletal development. Whereas de-repression of Gli3 appears to be the predominant mode through which Ihh controls chondrocyte proliferation and

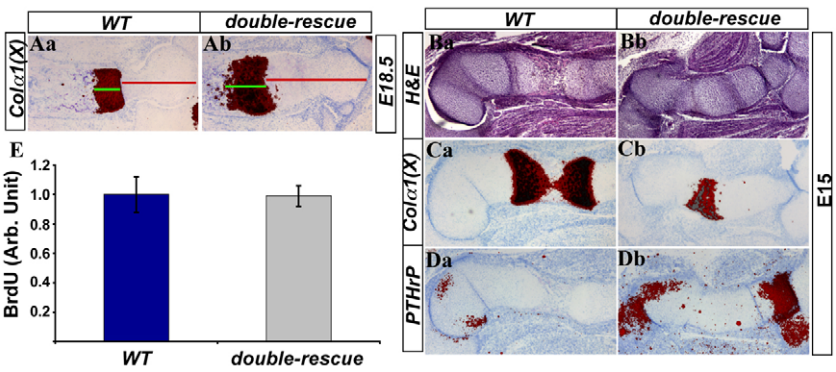

Fig. 6. Analysis of chondrocyte maturation and proliferation in double-rescue embryos. (**Aa**,**b**) In situ hybridization at E18.5. Distal end to the right. Red line, proliferative zone; green line, hypertrophic zone. (**Ba**,**b**) H&E staining at E15. (**Ca-Db**) In situ hybridization at E15. All sections longitudinal through the humerus; distal end to the right. (**E**) Relative BrdU labeling index for chondrocytes in the distal half of the humerus at E17.5. Results shown for one littermate pair, three sections counted for each embryo. P=0.91. Similar results were obtained at other stages.

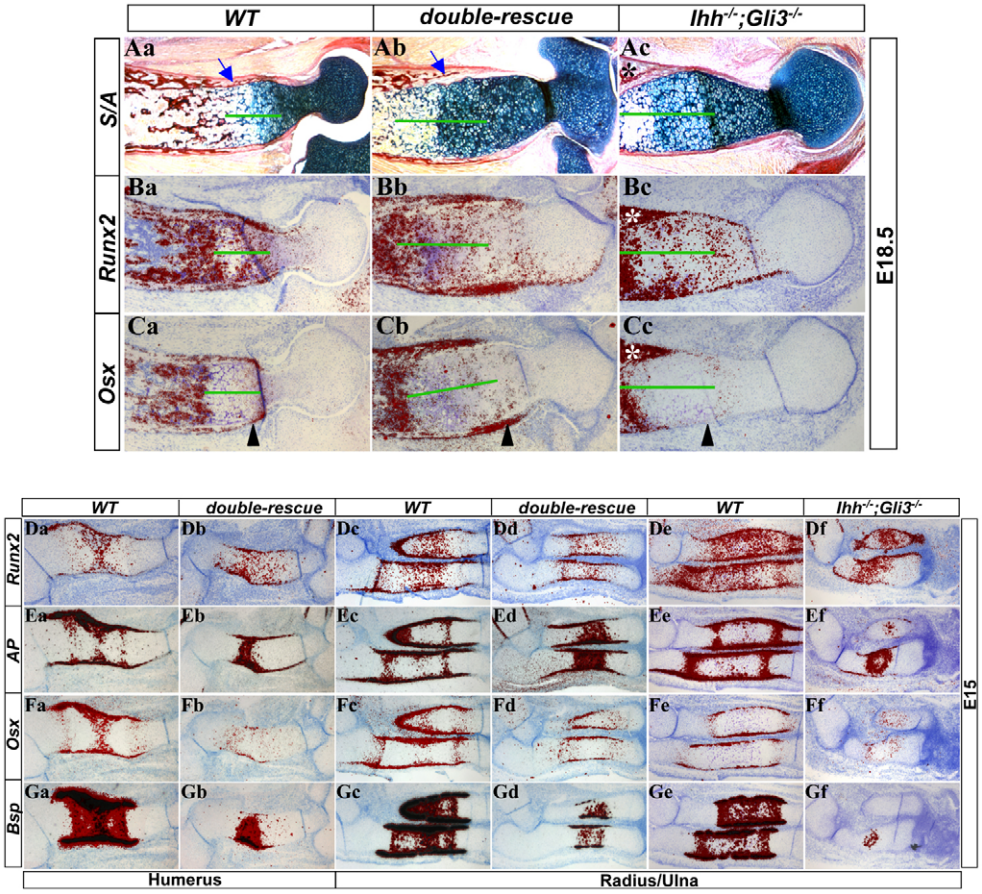

Fig. 7. Analysis of osteoblast differentiation. (**Aa-c**) Sirius Red and Alcian Blue staining at E18.5. (**Ba-Cc**) In situ hybridization on adjacent sections at E18.5. All sections longitudinal through the humerus; distal end to the right. Blue arrows, bone collar; asterisks, invading mesenchyme at diaphysis; green lines, hypertrophic zone; black arrowheads, perichondrium flanking the hypertrophic zone. (**Da-Gf**) In situ hybridization on adjacent sections at E15. Mutant embryos are shown along with their littermate wild-type embryos. Longitudinal sections through the humerus or the radius/ulna; distal end to the right.

hypertrophy, activation of Gli2 is likely to play a more important role in inducing vascularization of the hypertrophic cartilage (Fig. 8). Conversely, both regulatory modes are necessary for Ihh to induce osteoblast formation within the proper domain of the perichondrium, even though Gli3 de-repression alone is sufficient to produce *Runx2*-positive cells (Hilton et al., 2005). Lastly, both this and the previous work point to an as yet undefined vasculature-dependent signal that is capable of activating the osteogenic program.

DISCUSSION

The current study investigates the transcriptional effectors of Ihh signaling in endochondral skeletal development. Although Ihh is known to be a key regulator of the process, the mechanisms through which it fulfills a multitude of diverse functions are not fully understood. Because gene knockout studies of *Gli1/2/3* did not reproduce a skeletal phenotype similar to that of an *Ihh*-null mouse, it was not certain whether the Gli proteins mediated all Ihh functions. Here, we have provided genetic evidence that all major functions of Ihh in the developing skeleton are primarily achieved through Gli2 and Gli3 transcription factors.

The study does not rule out the possibility that Gli1 might also participate in the regulation. Gli1 itself is a direct transcriptional target of Gli activators, and its expression in the developing endochondral skeleton depends on Ihh. Although not detectable by in situ hybridization in $Ihh^{-/-};Gli3^{-/-}$ embryos, Gli1 was detected in double-rescue embryos (data not shown). As a sole activator, Gli1 could contribute to target gene activation and therefore amplify the transcriptional effect of ΔNGli2. However, it is not certain at present to what extent Gli1 is active in the absence of a Hh ligand, as its activity, when tested ectopically in *Drosophila*, was shown to depend on Hh (Aza-Blanc et al., 2000). A clear discernment for a potential Gli1 contribution in the rescue embryos would require future experiments to delete *Gli1* in these genetic backgrounds. Nonetheless, because *Gli1*-null embryos do not exhibit any obvious phenotype, and because any Gli1 function in the rescue embryo is

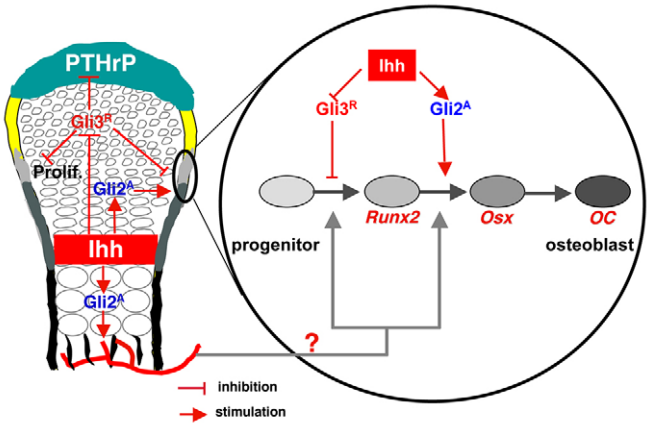

Fig. 8. A model for the Gli2 activator and the Gli3 repressor as effectors of Ihh signaling in endochondral bone development. Question mark denotes a speculated role for the vasculature in osteoblast differentiation. Arrow bifurcation indicates that the vasculature might regulate osteoblast differentiation at both steps.

secondary to the Gli2 activator, it is reasonable to suggest that Gli2 is the principal transcriptional activator in mediating Ihh function in the skeleton.

The transcriptional targets for Gli2 and Gli3 in the context of skeletal development remain to be elucidated. Although bone morphogenetic protein 2 (*Bmp2*) was reported to be a direct target of Gli2 in mediating osteoblast differentiation in a cell culture model (Zhao et al., 2006), its expression was retained in the perichondrium of $Ihh^{-/-}$ embryos (data not shown). Thus, *Bmp2* does not appear to be a crucial target gene through which Ihh controls osteoblast development in vivo. Likewise, no direct target genes have been identified with regard to cartilage hypertrophy or vascularization, although Pthrp expression critically depends on de-repression of Gli3 by Ihh (Hilton et al., 2005; Hilton et al., 2007), and expression of vascular endothelial growth factor (Vegfa) and of angiopoitin 1 and 2 is upregulated by Hh signaling in other systems (Lavine et al., 2006; Pola et al., 2001).

The mechanism through which Gli2 and Gli3 differentially control target gene expression also needs further investigation. The Gli2 activator and the Gli3 repressor could bind to distinct genomic sequences to regulate different sets of genes. However, this scenario is unlikely, as recent genome-wide analyses with the Gli1 activator and Gli3 repressor revealed little difference in DNA binding between the two proteins (Vokes et al., 2007; Vokes et al., 2008). Alternatively, Gli2 and Gli3 could compete to bind the same regulatory sequences, but removal of the Gli3 repressor, or binding of the Gli2 activator, could elicit different transcriptional responses in different genes. In other words, although certain genes might be activated in response to Gli3 removal, others might require binding of the Gli2 activator, and still others might require both.

In addition to its major functions in cartilage vascularization and osteoblast differentiation, the Gli2 activator appears to regulate chondrocyte maturation. In particular, ΔNGli2, when expressed in the wild-type background, promoted the onset of hypertrophy, without apparent reduction of Pthrp expression. However, when Pthrp was elevated above the wild-type level as it was in the double-rescue embryos, overall hypertrophy was delayed, indicating that Pthrp-mediated inhibition was dominant over the ΔNGli2-mediated acceleration. ΔNGli2 might also expedite the progression through different stages of hypertrophy following its onset, as this would explain the shortening of the hypertrophic zone in *C2ΔNGli2* embryos at E18.5. More importantly, when expressed in the $Ihh^{-/-}$ background, ΔNGli2 restored the normal separation of *Colα1(II)* and *Colα1(X)* expression domains, arguing that Ihh-Gli2 signaling might normally promote the progression of hypertrophy. Because hypertrophic chondrocytes are not known to respond to Ihh, such regulation could be secondary to the cells' earlier response to the signal.

Despite their clear differences, Gli2 activation and Gli3 de-repression appear to also regulate certain common aspects of skeletal development. For instance, the Gli2 activator is likely to play a minor role in Pthrp expression and chondrocyte proliferation as both parameters were partially restored in the ΔNGli2-rescue embryos, albeit to a much lesser degree than in the $Ihh^{-/-};Gli3^{-/-}$ embryos. Additionally, as with de-repression of Gli3, the Gli2 activator restored a columnar region where chondrocytes exhibited a flat morphology. Finally, regarding hypertrophic cartilage vascularization, although Gli2 activation appeared to exhibit a more prominent role than Gli3 de-repression, full normalization of vascularization only occurred in the double-rescue embryo. Thus, de-repression of Gli3 might cooperate with Gli2 activation to control vascularization of the hypertrophic cartilage.

The exact mechanism through which Ihh controls cartilage vascularization remains to be elucidated. The fact that Gli2 activation limited to chondrocyte and osteoblast lineage cells (by *Col2-Cre*) improved vascularization indicates that a Gli2-dependent secreted molecule might be responsible for regulating endothelial cell behavior. The nature of such a molecule is presently unknown. Despite the documented role of Vegf in cartilage vascularization (Zelzer et al., 2002), it does not appear to be the prime mediator in the present setting, as Vegf expression appeared to be normal in the hypertrophic chondrocytes of $Ihh^{-/-}$ embryos (data not shown).

The role of the vasculature in osteoblast differentiation warrants further research. Aside from the nutritional utility that the vasculature fulfills, signaling molecules could emanate from the blood vessels. The de novo formation of osteoblasts in the *Ihh*-null single-rescue embryos (either by Gli3 removal or by ΔNGli2 expression) was invariably coupled with vascularization in the diaphyseal region, lending credence to the notion that instructive signals might be derived from the vasculature. Such signals could induce osteoblast differentiation either completely independently of Ihh signaling, or in conjunction with Gli3 de-repression or Gli2 activation. In any case, identification of such signals represents a future challenge.

The apparent involvement of Gli2 and Gli3 in chondrocyte morphology is consistent with the reported roles of Ihh and Smo in this aspect (Hilton et al., 2007; Kobayashi et al., 2005), but the underlying mechanisms remain unknown. Of note, a recent study implicated Fzd7-mediated planar cell polarity (PCP) signaling in the formation of flat chondrocytes in the columnar region (Li and Dudley, 2009). It is tempting to speculate that Hh-Gli signaling might intersect with Wnt-Fzd pathways to control chondrocyte morphology.

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